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[Continued on next page]

(54) Title: ENDODERMAL PROGENITOR CELLS
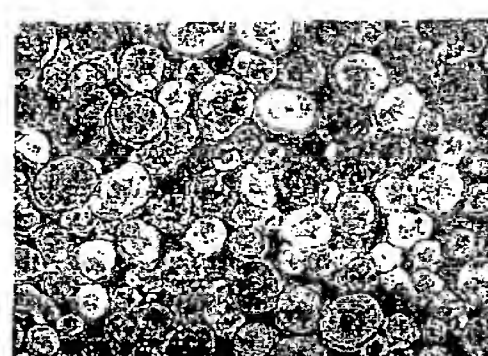
Day 0-2: Pancreatic cluster formation
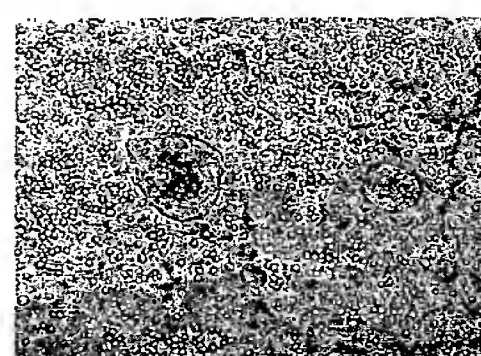
Day 10 and Beyond: Visible proliferation of PDPCs near GFP⁺ attached islet clusters
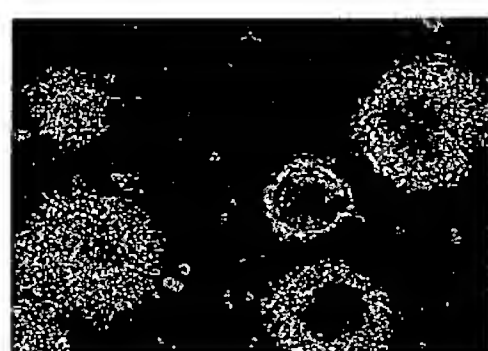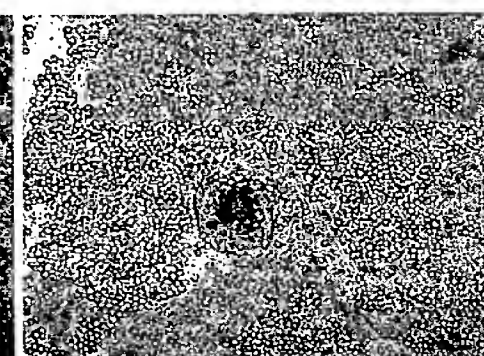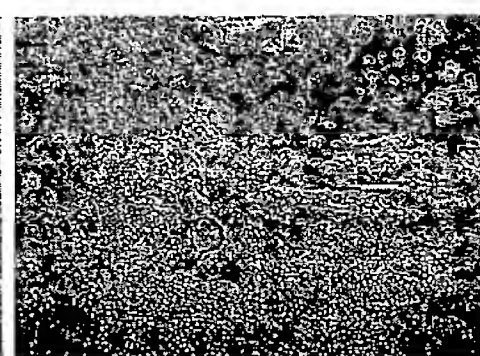
Culture in 2% FBS + EGF + Lif on FN coated dishes

Day 3: Attachment and spreading of clusters and islets
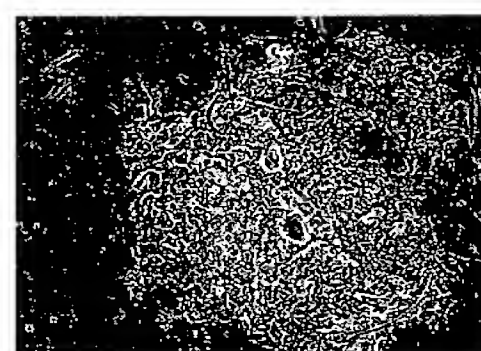
Day 5-10: Upon adherence and spreading of clusters, rapid proliferation of pancreatic mesenchymal layer without abundant PDPC proliferation was observed
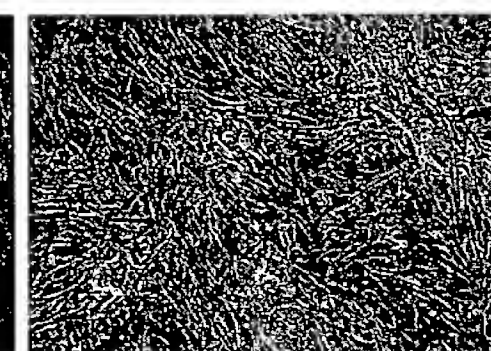
(57) Abstract: The present invention relates to endodermal progenitor cells and methods of isolation, culture, differentiation and use thereof.

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ENDODERMAL PROGENITOR CELLS

Related Application

5   This application claims priority from U.S. Provisional Application Serial No. 60/867,135 filed November 24, 2006, which application is herein incorporated by reference.

Statement of Government Rights

Field of the Invention

15   This invention relates to the field of progenitor cells obtained from endodermal tissues and methods of isolation, culture, differentiation and use thereof.

Background of the Invention

20   Type-1 diabetes (T1DM) is an autoimmune disorder which results in the selective destruction of more than 80% of endogenous insulin producing beta-cells (Pipeleers and Ling 1992). This disease most often appears during childhood or adolescence and is characterized by insulin deficiency, abrupt onset of symptoms, proneness to ketosis, and exogenous insulin dependency (Pociot
25   and McDermott 2002). It is estimated that as many as three million people in the U.S. suffer from T1DM (Juvenile Diabetes Research Foundation, 2004).

Although exogenous insulin delivery has remained a suitable remedy for most T1DM patients, some individuals continually suffer from uncontrollable blood glucose-levels, leading to disease-related complications including
30   blindness, neuropathy, nephropathy, myocardial infarction, stroke, and limb ischemia (Brownlee 2001). Over the last decade, islet transplantation has arisen as a potential alternative therapy for T1DM. Although islet-transplantation holds great promise, the scarcity of donor tissue makes such therapy available to <0.5% of potential transplant recipients.

A recent study has suggested that beta cells in the normal and regenerating adult murine pancreas are formed by self-duplication rather than contribution from a stem/progenitor cell (Dor et al. 2004). This data suggests a regenerative mechanism in the pancreas much like that described for the liver

5   (Michalopoulos and DeFrances 1997). Under steady state conditions to moderate damage (damage ≤ 2/3 cellular mass), hepatocytes proliferate as a means to replace damaged cells (Michalopoulos and DeFrances 1997; Oh et al. 2002). It is not until this hepatic damage is followed by inhibition of hepatocyte proliferation (i.e., 2-AAF treatment), or severe loss of hepatocytes (90%), that a

10   population of liver progenitor cells (termed oval cells) becomes activated and serves as a precursor for mature hepatocytes (Oh et al. 2002). Oval cells in the liver are bipotential progenitor cells, capable of giving rise to both hepatocytes and bile duct cells (Oh et al. 2002; Knight et al. 2005). Although not as extensively studied, oval cells have also been observed in the pancreas of rats

15   and mice fed a copper-deficient diet (Ide et al. 1993; Jeffers et al. 1996). Phenotypically, mouse liver oval cells have been described as expressing numerous epithelial and hematopoietic markers including alpha fetoprotein (Afp), cMet, cytokeratins (CKs) 7, 8, 18, 19, CD34, CD45, Sca1, and Thy1(Knight et al. 2005; Jeffers et al. 1996; Petersen et al. 2003; Petersen et al.

20   1998).

Cellular therapy with stem cells and their progeny is a promising medical treatment. Pancreatic islet transplantation provides a viable method for repopulation of pancreatic tissues damaged by disease or loss of pancreatic mass; however, current methods for culturing and maintenance of primary pancreatic

25   culture are not sufficient for successful transplantation. Isolation and maintenance of pancreatic oval cells requires the addition of chemicals or other exogenous stressors (e.g., *in vivo*) for their proliferation making the cells less useful clinically (Jeffers et al. 1996; Petersen et al. 2003; Petersen et al. 1998; Wang et al. 2003; Rao and Reddy 1991). Therefore, a need exists to identify,

30   isolate and characterize primitive cells that can differentiate into viable endodermal tissue, such as pancreatic tissue, and can be used in cellular replacement therapies, as well as to develop methods for isolating and culturing such cells without the need for the addition of chemicals or other exogenous stressors that may reduce the clinical applications of such cells.

Summary of the Invention

The present invention provides for the isolation of endodermal progenitor cells from tissue, such as endodermal tissue, including pancreas, which has not

5   been subjected to an exogenous stressor (e.g., *in vivo*). Methods of the invention can result in the isolation of a population of progenitor cells that have a different phenotype from other previously identified progenitor cells. Therefore, the present invention relates to a progenitor cell and methods of isolation, culture, differentiation and use thereof.

10   One embodiment provides a method of obtaining endodermal progenitor cells comprising: (a) culturing a cell population obtained from a tissue to induce cluster formation in culture medium; (b) culturing the clusters in culture medium; and (c) isolating endodermal progenitor cells, wherein the function of a cell in the tissue is not reduced by application of an exogenous stressor prior to

15   harvesting the tissue (e.g., the tissue has not been subject to any stressor while *in vivo*, including, but not limited to, physical manipulation, exposure to toxins or other agents, or subject to stress, such as nutritional stress (e.g., copper depletion diet) prior to harvesting).

In one embodiment, the tissue is endodermal tissue. For example, in one

20   embodiment, the endodermal tissue is derived from the group consisting of liver, stomach, intestine, pancreas, lung, colon, bladder or thyroid. In one embodiment, the cell population can be enriched or unfractionated (e.g., for pancreas, one can use an islet enriched fraction or unfractionated pancreas).

In another embodiment, the clusters are isolated prior to culturing in step

25   (b). In one embodiment, the clusters of step (b) are cultured with one or more attachment factors, including, but not limited to, one or more of collagen type I, collagen type II, collagen type IV, fibronectin, chondroitin sulfate, vitronectin, thrombospondin, or matrigel.

In one embodiment, the culture medium of step (b) is changed (e.g.,

30   replaced by fresh media) every two to three days for at least about 7 days. In another embodiment, after about 7 days the culture medium of step (b) is not changed prior to isolating the endodermal progenitor cells from the cultured population.

In one embodiment, the culture medium of step (a) and/or (b) comprises serum (e.g., about 0.5% to about 5% serum). In one embodiment, the culture medium of step (a) and/or (b) comprises one or more of EGF, LIF, basic FGF, or PDGF.

5   In one embodiment, the cell population is subjected to continuous density gradient centrifugation prior to step (a). In another embodiment, the tissue is dissociated (e.g., mechanically or chemically (e.g., enzymatically) so as to allow cells to dissociate from the tissue which cells can then be cultured so as to form clusters) prior to step (a).

10   In one embodiment, the endodermal tissue is disassociated enzymatically (e.g., by one or more of collagenase, trypsin, dispase I, hyaluronidase, thermolysin, neutral protease, liberase RI, DNase I, pancreatin, pronase or combination thereof). In one embodiment, the enzyme does not adversely effect the viability of the cell or enrich for a specific cell type.

15   In one embodiment, the tissue is obtained from a mammal (e.g., human, swine, rat or mouse, such as one which is free of diseases of the tissue of interest (e.g., liver disease (hepatitis), diabetes).

One embodiment provides an endodermal progenitor cell prepared by the methods described herein. In one embodiment, the progenitor cell originates in 20   the bone marrow. Another embodiment provides a composition (e.g., a pharmaceutical and/or cell culture composition) comprising a population of endodermal progenitor cells. In embodiment, the composition comprises a carrier (e.g., a pharmaceutically acceptable carrier and/or cell culture medium).

One embodiment provides a method for differentiating the endodermal 25   progenitor cells to yield an endodermal cell type (e.g., a cell type further differentiated than the endodermal progenitor cell, such as a cell that is committed to a particular endodermal cell type, including pancreatic, islet, intestinal, thyroid, lung, colon, bladder or liver cell types).

In one embodiment, the endodermal progenitor cells are differentiated 30   (*ex vivo* or *in vivo*) in the presence of one or more differentiation factors comprising one or more of β-cellulin, GLP-1, HGF, FGF, EGF, KGF, nicotinamide, TGF-α, TGF-β, activin, cyclopamin, BMP4, an SHH antibody, FGF, EGF, oncostatin M, dexamethasone, exendin4, GDF11, or nicotinamide.

One embodiment provides a method for providing an endoderm cell type to a subject in need thereof, comprising administering to the subject an endodermal progenitor cell described herein or differentiated progeny derived therefrom in an amount effective to provide an endoderm cell type to the subject.

5   Another embodiment provides a method wherein the endodermal progenitor cells or differentiated progeny thereof are administered by contacting the cells with a damaged tissue of the subject, such as a damaged liver or pancreas, but can also be contacted with normal tissues. An effective amount of endodermal progenitor cells or differentiated progeny therefrom can be administered to a

10   subject by systemic or localized injection, catheterized delivery and/or topical application.

One embodiment provides for use of endodermal progenitor cells in medical therapy. The medical therapy includes treating pancreatic, intestinal, thyroid, lung, bladder or liver damage as a result of an injury or disease.

15   Another embodiment provides the use of endodermal progenitor cells to prepare a medicament for treating pancreatic, intestinal, thyroid, lung, bladder, or liver damage as a result of injury or disease. The medicament may also include a physiologically acceptable carrier and/or cell culture medium.

20   **Brief Description of the Drawings**

Figure 1 depicts the PCR primers used for real-time RT-qPCR.

Figure 2 depicts the emergence of endodermal progenitor cells in culture initiated with islet-enriched preparations from Pdx-1-GFP mice. After cluster attachment, endodermal cells spread, followed by the proliferation of

25   mesenchymal cells (d5-10), and subsequent proliferation of pancreas-derived progenitor cells (PDPCs) surrounding attached islet clusters (green = Pdx1-GFP). Representative example of > 50 isolations.

Figure 3 depicts the characterization of PDPCs by immunohistochemistry, FACS and real-time qRT-PCR which reveal

30   coexpression of hematopoietic, endodermal and endothelial markers. (A) Day 14, PDPC cultures initiated with MIP-GFP pancreata were stained on day 14 with anti-CD45 (Alexa 594, yellow, panel b). $CD45^+$ cells are GFP negative (panel b and panel c = merge a+b). The mesenchymal cells stained positive with anti-smooth muscle actin (Alexa 594, red, panel e). Cells were counterstained

with DAPI. White arrows (panel d and e) show small round PDPCs growing on top of the smooth muscle actin$^+$ mesenchymal cells. Figures a-e = 20X. Stainings are representative of 2 independent experiments. (B) Cells were harvested from PDPC cultures on d12-14 and labeled with anti-CD45, Sca1,

5   CD34, Thy1, Mac1 and CD115 antibodies. Data shown is representative of 2 independent experiments. (C and D) Real-time qRT-PCR results on immunomagnetic purified CD45$^+$ cells (pooled RNA from three independent isolations; purity 95.2 ± 0.84%) (C) or FACS purified CD45$^+$Sca1$^+$/CD45$^+$Sca1$^-$ cells (D) from day 12-14 PDPC cultures. Shown is relative expression

10   compared to a primary F2 hepatic oval cell-containing fraction. ΔCt = Ct (Gene-of-Interest) – Ct (Reference Gene). Expression Levels: ++++ = ΔCt = < 6, +++ = ΔCt = 6-12, ++ = ΔCt =12-18, + = ΔCt = > 18. ND = Not Detected, NT = Not Tested.

Figure 4 depicts PDPCs differentiated for 4-6 weeks with high serum,

15   HGF, and dexamethasone. (A) Light microscopy of CD45$^+$ PDPCs (94-98% pure) cultured for 4-6 weeks on type I collagen in medium containing 10% FCS, 20ng/mL HGF and dexamethasone. (B) Real-time qRT-PCR of cells at the time of seeding (d0) and on cells harvested after 4-6 weeks in differentiation conditions. Results are expressed relative to mouse total liver except * = relative

20   to mouse total pancreas. Differentiation data represents average results of two independent differentiations. ΔCt = Ct (Gene-of-Interest) – Ct (Reference Gene). Expression Levels: ++++ = ΔCt = < 6, +++ = ΔCt = 6-12, ++ = ΔCt =12-18, + = ΔCt = > 18. *** = Marker analyzed on only n-1 differentiation. (C) Immunohistochemistry of PDPCs cultured under differentiation conditions for 3-

25   4 weeks. Control (d, e, f) is the Hepa1-6 hepatoma cell line. (a and d) isotype control, (b and e) Hnf3β-DAB (20X), (c and f) Hnf3β-DAB (40X).

Figure 5. PDPCs are not of an endodermal pancreatic origin. (A) PCR-based detection of lineage tracing employs a common forward CAAGS primer (black arrows) and specific reverse primers (blue, LacZ; green, GFP), allowing

30   detection of Z/EG reporter excision events. (B) Pancreata from Ins2-Cre x Z/EG (ZI) double transgenic animals constitutively express GFP in mature beta-cells (a) and pancreata from Pdx1cre x Z/EG (ZPC) double transgenic animals display GFP labeling in all pancreatic cell types (c). Under PDPC culture conditions for 14 days, neither ZI (b) nor ZPC (d) derived PDPCs were GFP positive. (C)

PDPCs harvested on d14 from cultures initiated with pancreata from ZI (a) or ZPC (b) animals, labeled with anti-CD45-PE and analyzed by FACS.

Figure 6. PDPCs are derived from the hematopoietic stem cell enriched KSL fraction of bone marrow. (A) Total BM (a) or KSL cells (c) from Actb-GFP mice were transplanted in lethally irradiated Rag2γc$^{-/-}$ mice. Three months later, pancreata were harvested. GFP$^+$ cells could be seen predominantly in islet clusters (a, c). Pancreata were cultured under PDPC conditions and evaluated on d12-14 by immunofluorescence (b, d). The majority of PDPCs were GFP$^+$. Representative example of 3 independent isolations each for total BM and KSL experiments. (B) PDPCs were harvested on day 14 from cultures initiated with pancreata from GFP$^+$ BM (a) or KSL (b) grafted animals and labeled with anti-CD45-PE and analyzed by FACS.

Figure 7. PDPCs are not the result of BM-derived cells fusing with pancreas cells. (A) The islet-enriched fraction of pancreata from Z/EG animals grafted with BM from Actbcre did not show visible GFP$^+$ cells at d0 (a). When cells were cultured for 14 days under PDPC culture conditions, PDPCs were not GFP$^+$ (b). (B) PDPCs were harvested from cultures on day 14 and labeled with anti-CD45-PE and confirmed by FACS. (C) DNA was obtained from FACS purified CD45$^+$ PDPCs isolated from grafted animals and PCR was performed using CAAGS-LacZ and CAAGS-GFP and Cre primers to detect excision and the presence of Actbcre-derived CD45$^+$ PDPCs.

Figure 8. Bone marrow cells do not acquire PDPC characteristics by coculture with pancreatic cells. GFP$^+$Sca1$^+$ BM cells were spiked into day 7 PDPC cultures initiated with pancreata from wild-type animals. (A) 7 days later, GFP positive cells were present throughout apparent PDPC colonies as demonstrated by fluorescence microscopy. (B) Cells were harvested on day 14 of culture, stained with anti-CD45 antibodies and analyzed by FACS. (C) GFP$^+$CD45$^+$ cells were sorted by FACS (B-b, c) and analyzed by real-time RT-qRT-PCR for CK7, CK8, CK18, CK19 and Afp in comparison with levels in freshly isolated Sca1$^+$ BM cells. Expression relative to fresh Sca1$^+$ cells and CD45$^+$ PDPCs is shown.

Detailed Description of the Invention

Definitions

As used herein, the terms below are defined by the following meanings:

" Endodermal progenitor cells," "Pancreas-derived endodermal
progenitor cells," "Pancreas-derived progenitor cells" or "PDPCs" are cells
isolated from an endodermal tissue (e.g., pancreas) that can differentiate into
more than one endodermal cell type (such cells may also be able to differentiated
into cells of more than one embryonic layer (e.g., endodermal, mesodermal,
and/or ectodermal)).

The term "isolated" refers to a cell or cells which are not associated with
one or more cells or one or more cellular components that are associated with the
cell or cells *in vivo*.

A "cluster" of cells is defined herein as a multi-cellular aggregate
consisting of more than a single cell (e.g., when cultured in suspension) that has
initially developed from a single or from multiple cells. For example, a cluster
of cells is an aggregate of more than one cell, including two or more cells, about
5 cells, about 10 cells, about 20 cells, about 25 cells, about 30 cells, about 35
cells, about 40 cells, about 50 cells, about 60 cells, about 70 cells, about 80 cells,
about 90 cells about 100 cells, about 150 cells, about 200 cells and so on (e.g., a
cluster of cells can include an aggregate of cells comprising about 10 to about
100 cells or about 50 to about 1000 cells or greater). Clusters can be grown
adherently or in suspension.

A "subject" is a vertebrate, such as a mammal, including a human.
Mammals include, but are not limited to, humans, farm animals, sport animals
and companion animals. Included in the term "animal" is dog, cat, fish, gerbil,
guinea pig, hamster, horse, rabbit, swine, mouse, monkey (e.g., ape, gorilla,
chimpanzee, orangutan) rat, sheep, goat, cow and bird. Subjects that can benefit
from the cells and methods of the invention include, but are not limited to, those
suffering from a loss of function of endodermal cells, including, but not limited
to, liver or pancreatic cells, as a result of physical or disease related damage.

An "effective amount" generally means an amount which provides the
desired local or systemic effect and/or performance, particularly for treating a
condition of interest.

The terms "comprises," "comprising," and the like can have the meaning ascribed to them in U.S. Patent Law and can mean "includes," "including" and the like. As used herein, "including" or "includes" or the like means including, without limitation.

5   Isolation and Characterization of Pancreas-Derived Endodermal Progenitor Cells

The present invention relates to endodermal progenitor cells and methods of isolation, culture, differentiation and use thereof. Unlike traditional isolation and culture protocols for liver or pancreatic oval cells, methods of the present invention require no exogenous stressor (e.g., reduction of cell mass and/or

10   function) to the endodermal tissue prior to or during harvesting of the endodermal tissue. As used herein, an "exogenous stressor," includes adverse chemicals (*e.g.*, those which render a cell preparation unsuitable for clinical use), toxins (e.g., N-2-acetylaminofluorene, 3,5-diethoxycarbonyl-1.4-dihydrocollidine, streptozotocin), nutritional stressors (e.g., diets deficient in one

15   or more nutrients or diets supplemented with an agent (e.g., an agent that effects cell function or alters cell environment)), cell depletion, or physical stress. For example, the subject/endodermal tissue, such as liver or pancreas, has not been manipulated/exposed to surgical resection, pancreatectomy (e.g., partial), pancreatic duct ligation, nutritional stress (e.g., copper depleted diet, choline-

20   deficient diet), or necrosis/atrophy, chemically induced or otherwise, prior to harvesting of the endodermal tissue, or harvesting conditions designed to destroy cells (e.g., hepatocytes). (Preferably the enzymes used during harvesting do not adversely effect the viability of the cell or enrich or select for a specific cell type.) For example, prior to harvesting, pancreatic tissue is not depleted (a

25   reduction) of acinar tissue or cells. Frequently, cells isolated by protocols with exogenous stressors transform into a tumorigenic phenotype due to treatment agents etc., precluding their use for clinical application.

In one embodiment, the endodermal tissue has not been manipulated so as to stimulate endodermal tissue growth and/or inhibit cell growth, for example,

30   inhibit normal mechanisms for liver growth restoration, such as by exposure to toxins, for example, those toxins and/or agents which inhibit mature cells, including but not limited to, hepatocytes. For example, the endodermal tissue, such as liver or pancreas, is not manipulated to generate concurrent stimulation of growth and inhibition of normal mechanisms for tissue growth (i.e., blockade

of proliferation of hepatocytes). For example, the stimulus for tissue growth can be satisfied through different methods, including surgical resection, nutritional stress, or chemically induced necrosis. Blockade of proliferation is frequently achieved using chemicals (such as 2-acetylaminofluorene and various

5   carcinogens) that impede or prevent mitotic division of mature endodermal cells (e.g., hepatocytes).

Methods of isolating endodermal progenitor cells are provided including disassociating an endodermal tissue to form a heterogeneous population of primary cells (e.g., pancreatic cells); culturing the primary cell population in

10   liquid culture medium so that cells in the primary cell population form clusters; culturing the clusters in culture medium in the presence of one or more attachment factors and isolating the endodermal progenitor cells.

Starting material for the culture system comprises heterogeneous populations of primary endodermal cells which can be obtained according to any

15   method available to the art, including, but not limited to, enzymatic degradation, mechanical separation, filtration, centrifugation (e.g., continuous density gradient (e.g., sucrose, Percoll, or Ficoll Hypaque) centrifugation, differential centrifugation) and combinations thereof. Additionally, immunomagnetic beads can also be used to selectively separate cell populations of interest based on cell-

20   surface marker expression. The endodermal tissue can be obtained from a mammal, such as rat, mouse, pig or human.

The number and quality of the isolated endodermal cells can vary depending on the quality of the tissue used, the composition of solutions, and the type and concentration of enzyme. Frequently used enzymes include, but are not

25   limited to, collagenase, pronase, trypsin, dispase I, hyaluronidase, thermolysin, neutral protease, liberase RI, DNase I, pancreatin or a combination thereof.

One embodiment provides a method of isolating a population of endodermal progenitor cells comprising: (a) disassociating harvested endodermal tissue to form a heterogeneous population of primary endodermal cells; (b)

30   culturing the primary cell population in culture medium so as to induce cluster formation; (c) culturing the clusters in culture medium in the presence of one or more attachment factors; and (d) isolating endodermal progenitor cells, wherein the endodermal tissue is not subject to an exogenous stressor prior to harvesting. In one embodiment, the primary endodermal cells are subjected to continuous

density gradient centrifugation prior to step (b). In one embodiment, the culturing of step (c) is extended for about 5 to about 7 days or greater, such as about 7 to about 14 days, or about 10 to about 14 days. Cultures can be extended for durations beyond 14 days, such as about 15 to about 20 days and

5   even longer, for about 21 to about 28 days, where desired.

The cells can also be isolated in the presence of various factors such as LIF (e.g., at a concentration of about 5,000 U/ml to about 50,000 U/ml, such as about 10,000 U/ml), EGF (e.g., at a concentration of about 5 ng/ml to about 50 ng/ml, such as about 10 ng/ml), basic FGF (e.g., at a concentration of about 5

10   ng/ml to about 50 ng/ml, such as about 10 ng/ml) or PDGF (e.g., at a concentration of about 5 ng/ml to about 50 ng/ml, such as about 10 ng/ml).

During and after isolation, the endodermal progenitor cells of the invention can be cultured in culture medium that is well established in the art and commercially available from the American Type Culture Collection

15   (ATCC). Such media include, but are not limited to, Dulbecco's Modified Eagle's Medium (DMEM), DMEM F12 medium, Eagle's Minimum Essential Medium, F-12K medium, Iscove's Modified Dulbecco's Medium, or RPMI-1640 medium. It is within the skill of one in the art to modify or modulate concentrations of media and/or media supplements as needed for the cells used.

20   It will also be apparent that many media are available as low-glucose formulations, with or without sodium pyruvate.

Also contemplated is supplementation of cell culture medium with mammalian sera. Sera often contain cellular factors and components that are necessary for viability and expansion. Examples of sera include fetal bovine

25   serum (FBS), bovine serum (BS), calf serum (CS), fetal calf serum (FCS), newborn calf serum (NCS), goat serum (GS), horse serum (HS), human serum, chicken serum, porcine serum, sheep serum, rabbit serum, rat serum (RS), serum replacements, and bovine embryonic fluid. It is understood that sera can be heat-inactivated at 55-65°C if deemed necessary to inactivate components of the

30   complement cascade. Modulation of serum concentrations, or withdrawal of serum from the culture medium can also be used to promote survival of one or more desired cell types. In one embodiment, the endodermal progenitor cells are cultured in the presence of FBS /or serum specific for the species cell type. For example, endodermal progenitor cells can be isolated and/or expanded with total

serum (e.g., FBS) concentrations of about 0.5% to about 5% or greater including about 5% to about 15%. Concentrations of serum can be determined empirically.

5   Additional supplements can also be used to supply the cells with trace elements for optimal growth and expansion. Such supplements include insulin, transferrin, sodium selenium, and combinations thereof. These components can be included in a salt solution such as, but not limited to, Hanks' Balanced Salt Solution® (HBSS), Earle's Salt Solution®, antioxidant supplements, MCDB-201® supplements, phosphate buffered saline (PBS), N-2-

10   hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), nicotinamide, ascorbic acid and/or ascorbic acid-2-phosphate, as well as additional amino acids. Many cell culture media already contain amino acids; however some require supplementation prior to culturing cells. Such amino acids include, but are not limited to, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-

15   cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-inositol, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.

Antibiotics are also typically used in cell culture to mitigate bacterial, mycoplasmal, and fungal contamination. Typically, antibiotics or anti-mycotic

20   compounds used are mixtures of penicillin/streptomycin, but can also include, but are not limited to, amphotericin (Fungizone®), ampicillin, gentamicin, bleomycin, hygromycin, kanamycin, mitomycin, mycophenolic acid, nalidixic acid, neomycin, nystatin, paromomycin, polymyxin, puromycin, rifampicin, spectinomycin, tetracycline, tylosin, and zeocin.

25   Hormones can also be advantageously used in cell culture and include, but are not limited to, D-aldosterone, diethylstilbestrol (DES), dexamethasone, β-estradiol, hydrocortisone, insulin, prolactin, progesterone, somatostatin/human growth hormone (HGH), thyrotropin, thyroxine, and L-thyronine. β-mercaptoethanol can also be supplemented in cell culture media.

30   Lipids and lipid carriers can also be used to supplement cell culture media, depending on the type of cell and the fate of the differentiated cell. Such lipids and carriers can include, but are not limited to cyclodextrin (α, β, γ), cholesterol, linoleic acid conjugated to albumin, linoleic acid and oleic acid

conjugated to albumin, unconjugated linoleic acid, linoleic-oleic-arachidonic acid conjugated to albumin, oleic acid unconjugated and conjugated to albumin, among others. Albumin can similarly be used in fatty-acid free formulation.

5   Also contemplated is the use of feeder cell layers. Feeder cells are used to support the growth of fastidious cultured cells, including pancreas-derived endodermal progenitor cells. Feeder cells are normal cells that have been inactivated by γ-irradiation. In culture, the feeder layer serves as a basal layer for other cells and supplies cellular factors without further growth or division of their own (Lim and Bodnar 2002). Examples of feeder layer cells typically used

10   with liver cell cultures are hepatocytes and embryonic fibroblasts (Suzuki, A. et al. 2000), but can be any post-mitotic cell that is capable of supplying cellular components and factors that are advantageous in allowing optimal growth, viability, and expansion of endodermal progenitor cells. In some cases, feeder cell layers are not needed to keep cells in an undifferentiated, proliferative state,

15   as leukemia inhibitory factor (LIF) has anti-differentiation properties. Often, supplementation of a defined concentration of LIF is all that is necessary to maintain cells in an undifferentiated state.

Cells in culture can be maintained either in suspension or attached to a solid support, such as extracellular matrix components and synthetic or

20   biopolymers. Cells often require additional factors that encourage their attachment to a solid support (e.g., attachment factors) such as type I, type II, and type IV collagen, concanavalin A, chondroitin sulfate, fibronectin, "superfibronectin" and/or fibronectin-like polymers, gelatin, laminin, poly-D and poly-L-lysine, Matrigel™, thrombospondin, and/or vitronectin.

25   The maintenance conditions of cells can also contain cellular factors that allow cells, such as the endodermal progenitor cells of the invention, to remain in an undifferentiated form. It may be advantageous under conditions where the cell must remain in an undifferentiated state of self-renewal for the medium to contain epidermal growth factor (EGF), platelet derived growth factor (PDGF),

30   leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and combinations thereof. It is apparent to those skilled in the art that supplements that allow the cell to self-renew (e.g., to produce replicate daughter cells having differentiation potential that is identical to those from which they arose; a similar term used in this context is "proliferation"), but not differentiate should be

removed from the culture medium prior to differentiation. It is also apparent that not all cells will require these factors. In fact, these factors may elicit unwanted effects, depending on the cell type.

5   Endodermal progenitor cells can be selected based on the markers (gene and/or protein) described herein. Accordingly, positive selection methods can be used, either alone or together with the methods described above, to identify and/or isolate the cells of the invention. Methods of positive selection can include visual selection, using microscopy and/or other means of detection, including, but not limited to, immunoblotting, immunofluorescence, and/or

10   enzyme-linked immunosorbent assay. Other methods of positive selection can also include, but are not limited to, additional selective culture techniques (e.g., variable cell densities or amounts of CO_2), flow cytometry, RT-PCR, and/or microchip-based methods of cell separation.

Uses for Endodermal Progenitor Cells

15   Endodermal progenitor cells can be used for the generation of endodermal lineages, including but not limited to, liver, pancreas, islet cells, lung, intestine, colon, thyroid, bladder and stomach. For example, liver cells, including hepatocytes and biliary epithelium and pancreatic cells, including acinar cells, ductal cells, islet cells, such as α-cells, β-cells, δ-cells, can be

20   generated from the endodermal progenitor cells described herein. Stomach cells that can be generated include, but are not limited to, mucosal cells, parietal cells, chief cells, and gastric endocrine cells. Intestinal cells that can be generated, include, but are not limited to, epithelial cells and enteroendocrine cells. Thyroid cells, such as, but not limited to, follicular and parafollicular cells can

25   be generated from the endodermal progenitor cells of the present invention. Additionally, lung cells, such as mucosal cells of the airways, which include ciliary epithelium, mucosal cells, serous cells, and alveolar cells, such as those that produce surfactants can be generated from the endodermal progenitor cells of the present invention.

30   Therefore, one embodiment provides methods for providing epithelial cells, which can include, but are not limited to, liver epithelial cells, biliary ductal epithelial cells, lung epithelial cells, gastric epithelial cells, or bowel epithelial cells, comprising differentiating endodermal progenitor cells in the presence of differentiation factors and isolating the epithelial cells. The

differentiation factors can be, but are not limited to, HGF, FGF, TGFα, TGFβ, EGF, Oncostatin M, dexamethasone, and/or nicotinamide. Differentiation can occur *in vivo* or *ex vivo*.

5   The invention further provides methods for providing endodermal cells, which can be, but are not limited to, exocrine pancreatic, endocrine pancreatic, islet, thyroid, intestinal, colon, bladder and/or lung cells. The endodermal progenitor cells of the invention are differentiated in the presence of differentiation factors including, but not limited to, β-cellulin, GLP-1, HGF, KGF, nicotinamide, FGF, FGF-4, TGF-α, TGF-β, activin, cyclopamin, and/or

10   BMP inhibitors, and the differentiation can occur *in vivo* or *ex vivo*.

Endodermal progenitor cells of the invention can be induced to differentiate into one or more liver cell types in the presence of cytokines and growth factors, which can be liver-specific (Michalopoulos and DeFrances 1997). For example, the endodermal progenitor cells can be induced to

15   differentiate into hepatocytes. Hepatocyte growth factor (HGF), or scatter factor, is a well-known cytokine that promotes differentiation to a hepatocyte phenotype. Similarly, epidermal growth factor (EGF) has also been implicated in proliferation and differentiation of liver cells. Other cytokines commonly associated with hepatic differentiation and proliferation are tumor necrosis

20   factor-α (TNFα), transforming growth factor-α (TGF-α), insulin, IGF-1 and -2, the interleukins, such as but not limited, to IL-4, IL-6, IL-8, IL-9, and IL-13, chemokines, such as macrophage inflammatory protein (MIP-1α, MIP-1β), RANTES, monocyte chemoattractant protein-1 (MCP-1), the GRO family, platelet derived growth factor (PDGF), keratinocyte growth factor (KGF),

25   fibroblast growth factor-1, -2, and -4 (FGF), and norepinephrine (Leffert, H.L. et al, (1988)). Endodermal progenitor cells can be differentiated in the presence of HGF and/or FGF-4, but can also include beta-cellulin, GLP-1, HGF, KGF, nicotinamide, TGF-α, TGF-β, activin, cyclopamin, and BMP inhibitors, among others.

30   Endodermal progenitor cells and other fastidious cells can benefit from co-culturing with another cell type. Such co-culturing methods arise from the observation that certain cells can supply yet-unidentified cellular factors that allow the stem cell to differentiate into a specific lineage or cell type. These

cellular factors can also induce expression of cell-surface receptors, some of which can be readily identified by monoclonal antibodies. Generally, cells for co-culturing can be selected based on the type of lineage one skilled in the art wishes to induce, and it is within the abilities of the skilled artisan to select the appropriate cells for co-culture.

Methods of identifying and subsequently isolating differentiated cells from their undifferentiated counterparts can be carried out by methods well known in the art. Cells that have been induced to differentiate can be identified by selectively culturing cells under conditions whereby differentiated cells outnumber undifferentiated cells. These conditions include, for example, extending the amount of time that cells are grown in culture, such that survival of a desired cell type is encouraged. Many primary cells achieve senescence, and fail to divide, or die, after a period of time. Other conditions comprise modulating the type and concentration of serum, or culturing the cells in the presence or absence of growth factors and/or cytokines that induce differentiation to another cell type. Differentiation can also be advantageously achieved by modulation of serum concentrations, or withdrawal of serum from the culture. Other methods of inducing differentiation can include, but are not limited to, modulating the acidity of the culture medium, as well as the oxygen and carbon dioxide levels during culture.

Similarly, differentiated cells can be identified by morphological changes and characteristics that are not present on their undifferentiated counterparts, such as cell size, the number of cellular processes (i.e., formation of dendrites and/or branches), and the complexity of intracellular organelle distribution. Also contemplated are methods of identifying differentiated cells by their expression of specific cell-surface markers such as cellular receptors and transmembrane proteins. Monoclonal antibodies against these cell-surface markers can be used to identify differentiated cells. Detection of these cells can be achieved through fluorescence activated cell sorting (FACS), and/or enzyme-linked immunosorbent assay (ELISA). From the standpoint of transcriptional upregulation of specific genes, differentiated cells often display levels of gene expression that are different from undifferentiated cells. Reverse-transcription polymerase chain reaction (RT-PCR) can also be used to monitor changes in

gene expression in response to differentiation. In addition, whole genome analysis using microarray technology can be used to identify differentiated cells.

Accordingly, once differentiated cells are identified, they can be separated from their undifferentiated counterparts, if necessary. The methods of identification detailed above also provide methods of separation, such as FACS, preferential cell culture methods, ELISA, magnetic beads, and combinations thereof. One embodiment of the invention envisions the use of FACS to identify and separate cells based on cell-surface antigen expression. It is understood that the methods of identification and separation are not limited to analysis of differentiated cell types, but can also be used to identify undifferentiated cell types such as the endodermal progenitor cells of the invention.

Endodermal progenitor cells of the invention can also be used in cell replacement therapies. Endodermal progenitor cells can be administered to a tissue of interest in a subject to supplement functioning cells or replace cells, which have lost function. Alternatively, methods of providing differentiated cells are also contemplated, wherein the endodermal progenitor cells are differentiated in the presence of differentiation factors, isolated, and administered into or upon the body of a subject. In one embodiment, the differentiated cells are cells of the endodermal lineage, such as pancreas cells (e.g., beta cells).

Disease states characterized by loss of liver mass and/or function, and that could benefit from endodermal progenitor cells and methods of the invention include, but are not limited to, Alagille Syndrome, alcoholic liver disease (alcohol-induced cirrhosis), α-1-antitrypsin deficiency, autoimmune hepatitis, Budd-Chiari Syndrome, biliary atresia, Byler Disease, cancer of the liver, Caroli Disease, Brigler-Najjar Syndrome, Dubin-Johnson Syndrome, fatty liver, galactosemia, Gilbert Syndrome, Glycogen Storage Disease I, hemangioma, hemochromatosis, hepatitis A-G, porphyria, primary biliary cirrhosis, sclerosing cholangitis, tyrosinemia, acquired liver disorders due to viral infections and/or Wilson's Disease.

Epithelial cells derived from endodermal progenitor cells of the invention can be used in cell replacement therapy to treat or alleviate symptoms of several organ diseases. The cells can be used to treat or alleviate congenital liver disorders, for example, storage disorders such as mucopolysaccharidosis,

leukodystrophies, GM2 gangliosidosis; increased bilirubin disorders, for instance Crigler- Najjar syndrome; ammonia disorders such as inborn errors of the urea-cycle, for instance ornithine decarboxylase deficiency, citrullinemia, and argininosuccinic aciduria; inborn errors of amino acids and organic acids

5   such as phenylketoinuria, hereditary tyrosinemia, αl-antitrypsin deficiency; and/or coagulation disorders such as factor VIII and IX deficiency.

Epithelial cells derived from endodermal progenitor cells of the invention can also be used in cell replacement therapy to treat or alleviate symptoms of biliary disorders such as biliary cirrhosis and biliary atresia, as well as to treat or

10   alleviate symptoms of pancreas disorders. Further, pancreas epithelium can be made from the cells of the present invention, as well as β-cells. These cells can be used for the therapy of diabetes (subcutaneous implantation or intra-pancreas, intra-liver or kidney sub-capsular implantation). Further, the epithelial cells derived from endodermal progenitor cells of the present invention can also be

15   used in cell replacement therapy and/or gene therapy to treat or alleviate symptoms of gut epithelium disorders such as gut atresia, inflammatory bowel disorders, bowel infarcts, and bowel resection.

Exogenous factors (e.g., cytokines, differentiation factors and other factors) can be administered prior to, after or concomitantly with the endodermal

20   progenitor cells of the invention. For example, a form of concomitant administration would comprise combining a factor of interest in the culture media and/or pharmaceutically acceptable carrier prior to administration. Doses for administrations are variable, may include an initial administration followed by subsequent administrations; but nonetheless, can be ascertained by the skilled

25   artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

Endodermal progenitor cells of the invention or their progeny can be administered via localized injection, including catheter administration, systemic injection, localized injection, parenteral administration, or intrauterine injection

30   into an embryo.

A method to potentially increase cell survival, when introducing the cells into a subject in need thereof, is to incorporate endodermal progenitor cells or their differentiated progeny of interest into a biopolymer or synthetic polymer. Depending on the subject's condition, the site of injection might prove

inhospitable for cell seeding and growth because of scarring or other
impediments. Examples of biopolymer include, but are not limited to, cells
mixed with fibronectin, fibrin, fibrinogen, thrombin, collagen, and
proteoglycans. This could be constructed with or without included cytokines,

5 growth factors, differentiation factors or nucleic acid expression constructs.
Additionally, these could be in suspension, but residence time at sites subjected
to flow would be nominal. Another alternative is a three-dimensional gel with
cells entrapped within the interstices of the cell biopolymer admixture. Again,
differentiation factors, growth factors or cytokines could be included within the

10 cells. These could be deployed by injection via various routes described herein.

An parameter involved in the therapeutic use of endodermal progenitor
cells is the quantity of cells necessary to achieve an optimal effect. In current
human studies of autologous mononuclear bone marrow cells, empirical doses
ranging from 1 to 4 x 10^7 cells have been used with encouraging results.

15 However, different scenarios may require optimization of the amount of cells
injected into a tissue of interest. Thus, the quantity of cells to be administered
will vary for the subject being treated. In one embodiment, between 10^4 to 10^8,
more preferably 10^5 to 10^7, and most preferably 3 x 10^7 progenitor cells and
optionally, 50 to 500 µg/kg per day of a cytokine can be administered to a

20 human subject. However, the precise determination of what would be
considered an effective dose may be based on factors individual to each patient,
including their size, age, size tissue damage, and amount of time since the
damage occurred. Therefore, dosages can be readily ascertained by those skilled
in the art from this disclosure and the knowledge in the art.

25 Another parameter involved in the use of endodermal progenitor cells is
the purity of the population. Pancreas or liver cells, for example, comprise
mixed populations of cells, which can be purified to a degree sufficient to
produce a desired effect. Those skilled in the art can readily determine the
percentage of endodermal progenitor cells in a population using various well-

30 known methods, such as fluorescence activated cell sorting (FACS). Preferable
ranges of purity in populations comprising endodermal progenitor cells are about
1 to about 5%, about 5 to about 10%, about 10 to about 15%, about 15 to about
20%, about 20 to about 25%, about 25 to about 30%, about 30 to about 35%,
about 35 to about 40%, about 40 to about 45%, about 45 to about 50%, about 50

to about 55%, about 55 to about 60%, about 60 to about 65%, about 65 to about 70%, about 70 to about 75%, about 75 to about 80%, about 80 to about 85%, about 85 to about 90%, about 90% to about 95% or about 95 to about 100%. Purity of the cells can be determined according to the cell surface marker profile

5   within a population. Dosages can be readily adjusted by those skilled in the art (e.g., a decrease in purity may require an increase in dosage).

The skilled artisan can readily determine the amount of cells and optional additives, vehicles, and/or carrier in compositions to be administered in methods of the invention. Typically, any additives (in addition to the active cell(s) and/or

10   cytokine(s)) are present in an amount of 0.001 to 50 % (weight) solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt %, preferably about 0.0001 to about 1 wt %, most preferably about 0.0001 to about 0.05 wt % or about 0.001 to about 20 wt %, preferably about 0.01 to about 10 wt %, and

15   most preferably about 0.05 to about 5 wt %. Of course, for any composition to be administered to an animal or human, and for any particular method of administration, it is preferred to determine the toxicity, such as by determining the lethal dose (LD) and LD_{50} in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable

20   therein and timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

25   When administering a therapeutic composition of the present invention, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions and dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered

30   saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the

action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable

5   pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the cells.

       Sterile injectable solutions can be prepared by incorporating the cells

10   utilized in practicing the present invention in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired. Examples of compositions comprising progenitor cells of the invention include liquid preparations for administration, including suspensions; and, preparations for intramuscular or intravenous administration (e.g., injectable administration),

15   such as sterile suspensions or emulsions. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity

20   enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, which is incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

25   Compositions of the invention are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient

30   to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues.

       The choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g.,

liquid dosage form (e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

Solutions, suspensions and gels normally contain a major amount of

5   water (e.g., purified, sterilized water) in addition to the cells. Minor amounts of other ingredients such as pH adjusters (e.g., a base such as NaOH), emulsifiers or dispersing agents, buffering agents, preservatives, wetting agents and jelling agents (e.g., methylcellulose), may also be present. The compositions can be isotonic, i.e., they can have the same osmotic pressure as blood and lacrimal

10   fluid.

The desired isotonicity of the compositions of this invention may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes.

15   Viscosity of the compositions, if desired, can be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The

20   concentration of the thickener will depend upon the agent selected. The point is to use an amount, which will achieve the selected viscosity. Viscous compositions are normally prepared from solutions by the addition of such thickening agents.

A pharmaceutically acceptable preservative or cell stabilizer can be

25   employed to increase the life of the compositions. If preservatives are used, it is well within the purview of the skilled artisan to select compositions that will not affect the viability or efficacy of the endodermal progenitor cells as described herein.

Those skilled in the art will recognize that the components of the

30   compositions should be selected to be chemically inert. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

Compositions can be administered in dosages and by techniques well known to those skilled in the medical and veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the composition form used for administration (e.g., solid vs. liquid).

5   Dosages for humans or other mammals can be determined without undue experimentation by the skilled artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

Suitable regimes for initial administration and further doses or for sequential administrations also are variable and may include an initial

10   administration followed by subsequent administrations; but nonetheless, can be ascertained by the skilled artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

Endodermal progenitor cells described herein or their differentiated progeny can be genetically modified by introducing heterologous DNA or RNA

15   into the cell by a variety of recombinant methods known to those of skill in the art. These methods are generally grouped into four major categories: (1) viral transfer, including the use of DNA or RNA viral vectors, such as retroviruses, including lentiviruses (Mochizuki, H., et al., 1998; Martin, F., et al. 1999; Robbins, et al. 1997; Salmons, B. and Gunzburg, W.H., 1993; Sutton, R., et al.,

20   1998; Kafri, T., et al., 1999; Dull, T., et al., 1998), Simian virus 40 (SV40), adenovirus (see, for example, Davidson, B.L., et al., 1993; Wagner, E., et al., 1992; Wold, W., Adenovirus Methods and Protocols, Humana Methods in Molecular Medicine (1998), Blackwell Science, Ltd.; Molin, M., et al., 1998; Douglas, J., et al., 1999; Hofmann, C., et al., 1999; Schwarzenberger, P., et al.,

25   1997), alpha virus, including Sindbis virus (U.S. Patent No. 5,843,723; Xiong, C., et al., 1989; Bredenbeek, P.J., et al., 1993; Frolov, I., et al., 1996), herpes virus (Laquerre, S., et al., 1998) and bovine papillomavirus, for example; (2) chemical transfer, including calcium phosphate transfection and DEAE dextran transfection methods; (3) membrane fusion transfer, using DNA-loaded

30   membranous vesicles such as liposomes (Loeffler, J. and Behr, J., 1993), red blood cell ghosts and protoplasts, for example; and (4) physical transfer techniques, such as microinjection, microprojectile J. Wolff in "Gene Therapeutics" (1994) at page 195. (see J. Wolff in "Gene Therapeutics" (1994)

at page 195; Johnston, S.A., et al., 1993; Williams, R.S., et al., 1991; Yang, N.S., et al., 1990), electroporation, nucleofection or direct "naked" DNA transfer.

Cells can be genetically altered by insertion of pre-selected isolated DNA, by substitution of a segment of the cellular genome with pre-selected

5   isolated DNA, or by deletion of or inactivation of at least a portion of the cellular genome of the cell. Deletion or inactivation of at least a portion of the cellular genome can be accomplished by a variety of means, including but not limited to genetic recombination, by antisense technology (which can include the use of peptide nucleic acids or PNAs), or by ribozyme technology, for example.

10   Insertion of one or more pre-selected DNA sequences can be accomplished by homologous recombination or by viral integration into the host cell genome. Methods of non-homologous recombination are also known, for example, as described in U.S. Patent Nos. 6,623,958, 6,602,686, 6,541,221, 6,524,824, 6,524,818, 6,410,266, 6,361,972, the contents of which are specifically

15   incorporated by reference for their entire disclosure.

The desired gene sequence can also be incorporated into the cell, particularly into its nucleus, using a plasmid expression vector and a nuclear localization sequence. Methods for directing polynucleotides to the nucleus have been described in the art. For example, signal peptides can be attached to

20   plasmid DNA, as described by Sebestyen, et al. (1998), to direct the DNA to the nucleus for more efficient expression.

The genetic material can be introduced using promoters that will allow for the gene of interest to be positively or negatively induced using certain chemicals/drugs, to be eliminated following administration of a given

25   drug/chemical, or can be tagged to allow induction by chemicals (including but not limited to the tamoxifen responsive mutated estrogen receptor) in specific cell compartments (including, but not limited to, the cell membrane).

Any of the transfection or transduction techniques can also be applied to introduce a transcriptional regulatory sequence into endodermal progenitor cells

30   or progeny to activate a desired endogenous gene. This can be done by both homologous (e.g., U.S. 5,641,670) or non-homologous (e.g., U.S. 6,602,686) recombination (these patents are incorporated by reference).

Successful transfection or transduction of target cells can be demonstrated using genetic markers. The green fluorescent protein of *Aequorea*

victoria, for example, has been shown to be an effective marker for identifying and tracking genetically modified hematopoietic cells (Persons, D., et al., 1998). Alternative selectable markers include the β-Gal gene, the truncated nerve growth factor receptor, and drug selectable markers (including but not limited to NEO, MTX, hygromycin).

Endodermal progenitor cells of the invention can be used for many diverse clinical and pre-clinical applications, which can include, but are not limited to, use in toxicological or genomic screening methods, determination of levels of enzymes and coagulation factors, as well as treatment of the diseases disclosed herein. Endodermal progenitor cells of the invention can provide a variety of differentiated cultured cell types for high-throughput toxicological or genomic screening. The cells can be cultured in, for example, 96-well or other multi-well culture plates to provide a system for high-throughput screening of, for example, target cytokines, chemokines, growth factors, or pharmaceutical compositions in pharmacogenomics or pharmacogenetics.

Thus, the present invention provides for use of endodermal progenitor cells to detect cellular responses (e.g., toxicity) to bioactive (biologic or pharmacologic) agents, comprising contacting a culture of cells, or the differentiated progeny thereof, with one or more biologic or pharmacologic agents, identifying one or more cellular response to the one or more biologic or pharmacologic agents, and comparing the cellular responses of the cell cultures to the cellular responses of control cultures.

The invention also envisions a tissue-engineered organ, or portion, or specific section thereof, a tissue engineered device comprising a tissue of interest and optionally, cytokines, growth factors, or differentiation factors that induce differentiation into a desired cell type, wherein the endodermal progenitor cells are used to generate tissues including, but not limited to, pancreas, lung, liver, intestine, thyroid, endocrine, esophagus, colon, stomach, and gall bladder. Tissue-engineered organs can be used with a biocompatible scaffold to support cell growth in a three-dimensional configuration, which can be biodegradable. Tissue-engineered organs generated from the endodermal progenitor cells can be implanted into a subject in need of a replacement organ, portion, or specific section thereof. The present invention also envisions the use of the endodermal

progenitor cells or cells differentiated therefrom as part of a bioreactor, e.g., a liver assist device.

Homogenous organs, portions, or sections derived from the endodermal progenitor cells of the invention can be implanted into a host. Likewise, heterogeneous organs, portions, or sections derived from endodermal progenitor cells induced to differentiate into multiple tissue types can be implanted into a subject in need thereof. The transplantation can be autologous, such that the donor of the stem cells from which organ or organ units are derived is the recipient of the engineered tissue. The transplantation can be heterologous, such that the donor of the stem cells from which organ or organ units are derived is not that of the recipient of the engineered-tissue (e.g., allogeneic or xenogenic).

Once transferred into a host, the tissue-engineered organs can recapitulate the function and architecture of the native host tissue. The tissue-engineered organs will benefit subjects in a wide variety of applications, including the treatment of cancer and other diseases disclosed herein, congenital defects, or damage due to surgical resection.

Example

The following example is provided in order to demonstrate and further illustrate certain embodiments and aspects of the present invention and is not to be construed as limiting the scope thereof.

Expansion and Characterization of Pancreas-Derived Endodermal Progenitor Cells (PDPCs)

Materials and Methods

Transgenic Animals. For mouse experiments the following transgenic strains were used: Pdx1-GFP (Gioquiang Gu, Vanderbilt University, Nashville, TN), MIP-GFP (Manami Hara, University of Chicago, Chicago, IL), Actb-GFP (Makio Ogawa, Medical University of South Carolina, Charleston, SC), Ins2cre (Jackson Labs, Bar Harbor, ME), Pdx1cre (Pedro Herrera, University of Geneva), Actbcre (Jackson Labs), Z/EG (Jackson Labs), and Rag2γc$^{-/-}$ (Taconic Labs, Hudson, NY) (Cao et al. 1995). While Actb-GFP and Rag2γc$^{-/-}$ breeders were maintained as homozygotes, all other breeders were maintained as heterozygotes and transgenic offspring were genotyped by PCR using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) as per manufacturers conditions using the following cycling protocol: 1) 95°C – 2 min, 2) 95°C –

30sec, 59°C – 30sec, 72°C – 30sec (35 cycles), 3) 72°C – 10 min. PCR products were visualized on a 2% agarose gel. Primers used included the following:
GAPDH, F:CATGGCCTTCCGTGTTCCTA (SEQ ID NO:1),
R:CTGGTCCTCAGTGTAGCCCAA (SEQ ID NO:2; 151bp); Pdx1-GFP,

5   F:ATGAGCAAGGGCGAGGAACTGTTC (SEQ ID NO:3),
R:GTGTCACCTTCGAACTTGACTTC (SEQ ID NO:4; 353bp); Z/EG,
F:ACTATCCCGACCGCCTTACT (SEQ ID NO:5),
R:CTGTAGCGGCTGATGTTGAA (SEQ ID NO:6; 175bp); Ins2cre,
F:CTCTGGCCATCTGCTGATCC (SEQ ID NO:7),

10   R:CGCCGCATAACCAGTGAAAC (SEQ ID NO:8; 550bp); Pdx1cre,
F:ACTACATCTTGAGTTGCAGGC (SEQ ID NO:9),
R:CCTGTTTTGCACGTTCACCG (SEQ ID NO:10; 700bp); Actbcre,
F:CTTCAGGCGCGCGGTCTGG (SEQ ID NO:11), R:
GAGACGGAAATCCATCGCTC (SEQ ID NO:12; 452bp). For Cre

15   recombinase-mediated lineage tracing, Ins2cre and Pdx1cre and Z/EG offspring were bred and resultant double transgenic offspring were used in subsequent studies. All transgenic animals were bred and maintained under SPF conditions in partial autoclave cages. University of Minnesota Research Animal Resources housing and husbandry guidelines are adapted from requirements in the Animal

20   Welfare Act and The Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996) (Grossblatt 1996).

Isolation and Culture of Mouse Pancreatic Fractions.

1. Islet-Enriched Fraction: Adult mice (10-24 weeks) were anesthetized by an IP dose of Avertin (250mg/kg), after which the abdomen was opened. The

25   pancreas was perfused with 2.5mL Liberase RI (Roche, Indianapolis, IN) in HBSS (Mediatech, Herndon, VA) + 0.1mg/mL DNase I (Roche), 25mM HEPES (Mediatech) and digested at 37°C for 14min. Digested pancreata were washed 3X in HBSS + 10% FBS (HyClone, Logan, UT) and islets were purified using a continuous density gradient as described previously (Guo et al. 2003). Islet-

30   enriched preparations were 30-60% pure as determined by staining with Dithizone (Sigma, St. Louis, MO). The islet enriched fraction was cultured overnight in culture medium (CM) consisting of 60:40 low-glucose DMEM (Mediatech):MCDB201 (Sigma) + 2% FBS, 10ng/ml human EGF (Sigma), 10,000U/mL Lif (Chemicon, Temecula, CA), 10,000 I.U. Penicillin/mL, 10,000

μg/mL Streptomycin (Mediatech), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 25mM HEPES (Mediatech), 0.1mM 2-Mercaptoethanol (Invitrogen), 1mg/ml BSA (Sigma) in 60mm hydrophobic dishes (Sarstedt, Newton, NC) at 37°C and 5% CO_2. For PDPC expansion, islet-enriched fractions were seeded at 10-20 islets/cm^2 in tissue cultured treated dishes (BD Biosciences, San Jose, CA) coated with 10μg/mL fibronectin (Sigma). Complete media changes were done every 2-3 days for 1 week, after which media was not changed further.

     2. Unfractionated Pancreas: Adult mice (10-24 weeks) were anesthetized and pancreata perfused as described above for isolation of the islet-enriched fraction. Digested pancreata were cultured for two days in CM in hydrophobic flasks (Sarstedt). To improve cell viability, suspension cultures were washed after 24hrs with PBS + 1mg/ml BSA. For PDPC expansion, unfractionated pancreas was seeded at a cell pellet dilution of 1:750 v/v in CM. Complete media changes were made every 2-3 days for 1 week as done for islet-enriched cultures. Successful generation of PDPCs from islet-enriched or total pancreas fraction was > 75%.

     3. Differentiation of PDPCs: CD45$^+$ cells from 12-14 day islet-enriched fraction cultures were seeded at 7-10x10^4 cells/cm^2 on Type 1 collagen (10ug/cm^2)(Upstate, Charlottesville, VA) coated 48 well-plates (BD Biosciences). Cells were seeded in CM supplemented with 10%FBS + 20ng/ml HGF (Sigma), 100nM Dexamethasone (sigma), and 0.25X ITS (Sigma). Media changes were made every 7-14 days. Cells were analyzed after 3-6 weeks.

     RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR.
Total RNA (1x10^5-1x10^6 cells) was isolated using an RNeasy Micro Kit (Qiagen, Valencia, CA). Eluted RNA was treated with Turbo DNA-free according to the manufacturers protocol (Ambion, Austin, TX) and reverse-transcribed into cDNA using Superscript III with random hexamers (Invitrogen). As relative expression controls, mouse liver total RNA (Ambion), mouse pancreas mRNA (Clontech, Mountain View, CA), mouse liver F2 oval cell-containing fraction (X. Wang, University of Minnesota) or mouse total bone marrow was reverse transcribed similarly and used in real-time qPCR analysis. For real-time qPCR, 5-10ng cDNA was amplified using SYBR® Green PCR Master mix with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) under the following conditions: 1) 50°C – 2min 2) 95°C – 10min 3) 95°C –

15sec , 59°C – 60sec (40 cycles), 4) 95°C – 15sec, 59°C – 20sec, 95°C – 15sec
(Dissociation Curve Analysis). In addition to dissociation curve analysis, PCR
products were verified by gel electrophoresis. Primer used for real-time qPCR
are listed provided in Figure 1.

5   **FACS Analysis.** To analyze PDPCs and select sub-populations from
PDPC cultures, cells were harvested after 14-21 days using 0.25% trypsin
(Mediatech), washed with PBS + 2% FBS (HyClone), 1mM EDTA (Ambion)
(F2-PBS), and filtered through a 40μm cell strainer (BD Bioscience). For FACS
analysis, 1x10^5 cells were stained with anti-mouse CD45-APC(30-F11), CD45-

10   PE (30-F11), CD45-PE-Cy7 (30-F11), Sca1-PE (E13-161.7), CD34-
PE(RAM34), Thy1-PE(30-H12), Mac1-PE (M1/70) (BD Bioscience), or
CD115-PE (eBioscience, San Diego, CA) antibodies and analyzed using a
FACSCalibur flow cytometer (BD Bioscience).

         Selection of Subpopulations from PDPC Cultures. To isolate sub-

15   populations from PDPC cultures, cells were stained with CD45-APC, CD45-PE,
or Sca1-PE and selected using a FACSAria flow cytometer (BD Biosciences).
Alternatively, cells from PDPC cultures were labeled with anti-mouse CD45-PE,
CD45-FITC (104), or CD45-Biotin (104) (BD Biosciences) and purified using
the immunomagnetic EasySep™ PE selection kit (Stem Cell Technologies,

20   Vancouver, BC). Cells were analyzed for purity by FACS prior to use in
subsequent experiments.

         Immunofluorescence and Immunohistochemistry. To stain PDPC
cultures for CD45$^+$ cells, cultures were washed 2X with F2-PBS, incubated for
15 minutes with CD45-PE (BD Bioscience), followed by 2X wash with F2-PBS.

25   To stain PDPC cultures for smooth muscle actin, PDPC cultures were fixed
(15min) with 4% formalin, incubated (15 min) with 100mM glycine (Sigma) in
PBS, washed 3X PBS, incubated (15 min) with 0.1% Tween-20 (Sigma) in PBS,
washed 3X PBS, blocked (30 min) in 0.4% fish skin gelatin (Sigma) and stained
overnight (4°C) with monoclonal mouse anti-smooth muscle actin

30   (Sigma)(1A4). Following overnight incubation, cells were stained (45 min –
room temperature) with anti-mouse AlexaFluor 594 (Invitrogen) and washed 4X
(15 min) in 0.1% Tween in PBS. For immunohistochemistry on differentiated
samples, cells were fixed with 4% formalin (12min) and staining was performed
using the streptavidin-biotin-peroxidase method as per the manufacturer's

protocol (Dako, Glostrup, Denmark) with goat anti-Hnf-3β(P-19)(sc-9187)
(Santa Cruz Biotechnology, Inc, Santa Cruz, CA). As a positive staining
control, the Hepa1-6 hepatoma cell line (ATCC, Manassas, VA) was used.
These cells were maintained in high-glucose DMEM (Mediatech) supplemented
5   with 10% FBS (Hyclone). Hepa1-6 cells were fixed and stained using the same
protocol applied to differentiation cultures.

    Hematopoietic Chimeras. Bone marrow was harvested from 6-8 week
old Actb-GFP mice, red blood cells lysed with ammonium chloride (Stem Cell
Technologies, Vancouver, BC), and in some instances cells were labeled with
10   anti-mouse CD117(c-Kit)-APC (2B8), Sca1-PE (E13-161.7), hematopoietic
lineage cocktail-biotin (Gr-1 (RB6-8C5), CD11b (M1/70), CD4 (GK1.5), CD8a
(53-6.7), Ter-119 (Ly-76), and CD45R/B220 (RA3-6B2) followed by staining
with streptavidin-PerCp (BD Bioscience). GFP^+ KSL (c-Kit^+/Sca-1^+/lineage
marker⁻; Ikuta and Weissman 1992) cells were sorted on the FACSAria. Rag2γc⁻
15   ⁄⁻ immunodeficient recipient mice were sub-lethally irradiated with 750cGy
cesium and injected (intra-venous, tail vein) with 1×10^6 GFP^+ mononuclear bone
marrow cells or 1×10^4 GFP^+ KSL-HSCs along with 2×10^5 wild-type C57Bl6
mononuclear bone marrow cells. Peripheral blood samples of recipient mice
were taken at 2-3 months post-transplant and analyzed by flow cytometry for
20   CD45 and GFP to determine % $CD45^+$ hematopoietic engraftment. Positively
engrafted mice were used for subsequent experiments.

    To generate chimeric Cre-Z/EG mice, recipient Z/EG reporter mice were
lethally irradiated with 950 cGy cesium and injected (intra-venous, tail vein)
with 1×10^6 Actbcre mononuclear bone marrow cells. To determine engraftment,
25   peripheral blood samples from recipient mice were analyzed after 2 months post-
transplant and analyzed by PCR using Platinum Taq DNA Polymerase
(conditions as described above) using the following primer set: Cre,
F:CGTACTGACGGTGGGAGAAT (SEQ ID NO:13),
R:CCCGGCAAAACAGGTAGTTA (SEQ ID NO:14; 166bp).
30   **Molecular Evaluation of Cre Recombinase Lineage Tracing.**
Genomic DNA was isolated using the QIAamp DNA micro kit (Qiagen), and
Platinum Taq PCR was performed using the following excision specific PCR
primer sets: CAAGS-LacZ, F:GTTCGGCTTCTGGCGTGT (NO:15),
R:GTTGCACCACAGATGAAACG (SEQ ID NO:16; 750bp); CAAGS-GFP,

F:GTTCGGCTTCTGGCGTGT (SEQ ID NO:17),

R:GTAGGTCAGGGTGGTCACGA (SEQ ID NO:18; 500bp). Excision PCR

reaction condition were : 1) 95°C – 2 min, 2) 95°C – 30sec, 59°C – 30sec, 72°C

– 60sec (40 cycles), 3) 72°C – 10 min. PCR products were visualized on a 2.0%

5   agarose gel.

Results

Generation of Pancreas-Derived Endodermal Progenitor Cells

(PDPCs) from Pancreas. Mouse islet-enriched pancreas fraction or

unfractionated pancreas were cultured in suspension conditions for 2 days in low

10   serum containing medium supplemented with EGF and LIF to induce spheroid

(cluster) formation (Figure 2). Resultant clusters were seeded in the same media

onto fibronectin coated dishes and quickly adhered, spread, and formed colonies

of epithelial cells among cells with mesenchymal morphology. To facilitate the

expansion of the mesenchymal-like cells, the culture medium was changed every

15   2-3 days. After 1 week, the medium was no longer changed which limited

mesenchymal-like cell proliferation and supported the rapid appearance and

proliferation of a distinct round or oval-shaped cell population surrounding islet

clusters. This emerging population was termed pancreas-derived endodermal

progenitor cells or PDPCs. PDPCs could be generated from either the

20   unfractionated or the islet-enriched fractions, but isolation from unfractionated

pancreas yielded more PDPCs/mouse pancreas ($1\text{-}3\text{x}10^5$). Moreover, resultant

PDPCs were phenotypically indistinguishable (data not shown).

When cultures were initiated with pancreata from Pdx1-GFP (Gu et al.

2004) transgenic animals in which beta-cells are GFP positive, dense colonies of

25   PDPCs began to emerge on top of the layer of mesenchymal cells, with the most

abundant growth observed near adherent GFP^+ islet clusters (Figure 2).

However, few, if any, PDPCs were GFP-positive. Immunofluorescence staining

on late-stage PDPC cultures revealed that the round cell population was positive

for the pan-leukocyte marker CD45, while the adherent mesenchymal cells were

30   positive for smooth muscle actin (Figure 3A). Flow cytometry confirmed that

PDPCs were positive for CD45 as well as the hematopoietic stem/progenitor cell

markers CD34, Sca1, Thy1 and the monocyte markers Mac1 and CD115 (Figure

3B). PDPCs were, however, negative for the granulocyte, B-cell, and T-cell

lineage markers Gr1, B220, CD4 and CD8, respectively (data not shown).

Quantitative real-time PCR on CD45$^+$ enriched PDPCs revealed expression of additional hematopoietic stem/progenitor markers including Lmo2, Runx1, HoxB4, Scl (Tal1), Gata2, Ikaros, Kit, Blnk, PU.1, and Cxcr4 (not hematopoietic specific) (Figure 3C). Enriched CD45$^+$ PDPCs also expressed

5   significant levels of early endoderm/epithelial markers (Afp, Met, Ttr, Sox7, CKs 7, 8, 18, 19 and Cdh1) and endothelial markers (Flk1, Cdh5, CD31, and vWF) (Figure 3C, Figure 4B). Since many of these markers are also expressed by hepatic oval cells, expression of many of these transcripts in PDPCs were compared to a primary isolated mouse hepatic F2 oval cell-containing fraction, a

10   size purified fraction that has been observed to arise during DDC-induced mouse liver regeneration (Wang et al. 2003). Compared to the F2 fraction, PDPCs expressed higher levels of most hematopoietic transcripts and the early endodermal transcripts Afp and Sox7, but expressed lower levels of the epithelial transcripts CK7, CK19, Cdh1 and endothelial transcripts. The CD45$^+$

15   Sca1$^+$ purified sub-population expressed higher levels of CK7, CK19, and Cdh1, (60.71%, 12.59%, and 13.82%, respectively, compared to the F2 fraction) compared to only CD45$^+$ cells. (Figure 3D). Similar to oval cells, PDPCs were also found to produce numerous cytokines and growth factors including MCSF, MIP1β, MIP1γ, MIP2, MCP1, MCP3, MCP5, IL-6 and SCF, as determined by

20   ELISA and real-time RT-qPCR (data not shown) (Knight et al. 2005; Fujio et al. 1996).

Endoderm Differentiation of PDPCs. In late-stage cultures, PDPCs underwent morphological changes from small and round to a more flattened shape, suggesting that they might be able to differentiate to more mature

25   epithelial/endoderm cells. Therefore, CD45$^+$ immunomagnetic purified PDPCs (94-98% pure) (Figure 4 A-a) were cultured for 4-6 weeks on Type I collagen coated plates with 10% FBS and HGF, a factor previously shown to direct stem cells to a hepatic fate (Schwartz et al. 2002; Schwartz et al. 2005). During the first 7-10 days after plating, PDPCs adhered, spread, and began to proliferate.

30   After 1-2 weeks in culture, proliferation ceased and the vast majority (>80%) of the cells acquired a flattened morphology (Figure 3 A-b). Real-time RT-qPCR demonstrated a 20 to 130-fold increase in most epithelial transcripts (CK7, 8, 18, 19 and Cdh1) (Figure 4B) (n=2). In addition, many endodermal transcription factors (Hnf1β, Hnf3α, Hnf3β, Hnf4 and Hnf6), which were initially expressed

at low or undetectable levels, were up-regulated between 290 and 1,950-fold. Expression of Afp was significantly lower whereas levels of the mature hepatocyte transcripts Ttr, Albumin, Cyp2b13, and Cyp1a1 was significantly increased (Figure 4B). Compared to mouse liver, most epithelial markers and

5   many transcription factors (including Gsc, Sox17, Hnf1β and Hnf3β) were expressed at near equal or greater levels. Interestingly, expression of some pancreatic transcripts (Pdx1 and Nkx6.1) were also increased, but levels of Ins2 were decreased suggesting a lack of robust beta-cell differentiation. It is noteworthy that the expression level of CD45 remained relatively constant

10   during differentiation (Figure 4B), suggesting the results are not likely caused by the outgrowth of a small contaminating CD45⁻ sub-population.

The upregulation seen by real-time qPCR for Hnf3β (Figure 4C) was confirmed by immunohistochemistry on cells allowed to differentiate for 3-4 weeks (n=2). Staining results displayed areas of cultures with distinct nuclear

15   staining for Hnf3β (Figure 4-b, c), in a manner similar to that seen for control Hepa1-6 cells (Figure 4C-e, f). Positively stained cells displayed a flattened epithelioid morphology, with some visible binucleated cells (Figure 3C-b, c).

Pancreatic Lineage Tracing of PDPCs. To establish the origin of PDPCs, it was first tested whether they were derived from pancreatic endoderm

20   or possibly mature beta-cells. To address this, Pdx1cre-Z/EG (ZPC) and Ins2cre-Z/EG (ZI) double-transgenic lineage tracing animals were generated. These mice provide Pdx-1 or Ins2 promoter-specific expression of the bacteriophage P1 Cre-recombinase in addition to the Z/EG reporter construct. In agreement with previously published data (Postic et al., 1990; Gannon et al.

25   2000; Novak et al. 2000), in ZI animals islet-specific GFP-expression was seen (Figure 5B-a), while in ZPC animals all pancreatic cells were GFP positive (Figure 5B-c). In none of the ZI or ZPC cultures established were significant levels of GFP positive PDPCs detected as evaluated by direct immunofluorescence microscopy (Figure 5B-b,d) and FACS for CD45 and GFP

30   (Figure 5C-a,b). To exclude the possibility that the GFP expression was silenced, primer sets were designed which utilize a common CAAGS-specific forward primer with two unique reverse primers, one specific for GFP and the other LacZ (Figure 5A). PCR amplification using the CAAGS-LacZ primer set amplifies a product in the unexcised Z/EG reporter construct, while the CAAGS-

GFP primer set only amplifies the resultant excised construct. PCR on genomic DNA isolated from FACS-purified (>99%) CD45$^+$ ZPC PDPCs displayed amplification of only the CAAGS-LacZ PCR product (Figure 5D, lane 1). In addition, real-time qPCR of the same sample using the CAAGS-GFP primer set revealed only 0.002% excision as compared to an Actbcre-Z/EG (100%) excision control (data not shown). This very low level of excision is likely caused by low levels (<1%) of contamination during FACS purification.

Total Bone Marrow and KSL-HSC Lineage Tracing. As PDPCs express a large number of hematopoietic markers, it was tested whether they are bone marrow (BM) derived, as has also been suggested for hepatic oval cells (Petersen et al. 1999). Sublethally irradiated Rag2γc$^{-/-}$ recipient mice were reconstituted with total BM from Actb-GFP transgenic animals. Rag2γc$^{-/-}$ mice are defective in the recombinase activating 2 gene and are therefore unable to carry out V(D)J rearrangement. These mice fail to generate mature T or B lymphocytes, but otherwise have apparently normal hematopoiesis (Cao et al. 1995). Examination of the islet-enriched pancreatic fractions harvested from hematopoietic chimeras 3-months after transplantation, revealed that the vast majority of GFP$^+$ cells reside within the islet compartment (Figure 6A-a). Late-stage cultures (n=3) displayed robust, uniform GFP-labeling of PDPCs (Figure 6A-b), confirmed by FACS for CD45 and GFP (Figure 6B-a), indicating that PDPCs are derived from cells within the BM. FACS purified GFP$^+$KSL cells, enriched for hematopoietic stem cells, were grafted into irradiated Rag2γc$^{-/-}$ recipient mice (n=3). At 2-3 months post transplant, 91.20 ± 4.36% (% GFP$^+$ CD45$^+$ PDPC vs. % GFP$^+$ CD45$^+$ total BM) of PDPCs were CD45$^+$GFP$^+$ and hence derived from the GFP$^+$KSL BM-derived graft (Figure 6B-b) Although PDPCs have a KSL BM origin and a hematopoietic progenitor phenotype, they did not form hematopoietic colonies in a methylcellulose colony forming assay, a commonly employed assay for the determination of hematopoietic progenitor potential (data not shown).

Determination Whether Fusion Between Bone Marrow Cells and Pancreas Derived Cells Underlies the Generation of PDPCs. As fusion has been recognized as a mechanism for BM plasticity both *in vivo* (Wang et al. 2003) and *ex vivo* (Terada et al. 2002), it was tested whether PDPCs are derived from BM-derived cells that fuse with pancreatic cells *in vivo* or *ex vivo*. To test

this, recipient Z/EG reporter mice were lethally irradiated and injected with Actbcre total BM cells. As these cells provide ubiquitous expression of Cre-recombinase, resultant BM-derived PDPCs in this model will express Cre and will coexist with recipient Z/EG pancreatic cells. If PDPCs were derived from

5   fusion between BM-derived cells and pancreatic cells *in vivo* or *ex vivo*, expression of the Cre and Z/EG genes in the same cell should result in excision of the LoxP-flanked cassette and will result in GFP-fluorescence (Doyonnas et al. 2004). Primary islet-enriched fractions and late-stage PDPC cultures from these animals showed no detectable GFP-fluorescence in unpurified (bulk) and

10    $CD45^+$ enriched PDPCs (Figure 7A and B). Excision PCR was performed and displayed no evidence of excision in bulk and $CD45^+$ fractions (Figure 7C). It is noteworthy that significant levels of Cre genomic DNA was detected by PCR in the $CD45^+$ fraction, ensuring a significant presence of Cre donor-derived PDPCs (Figure 7C, lane 2). Real-time qPCR for the CAAGS-GFP primer set revealed

15   0.23% excision in the $CD45^+$ fraction and 1.96% excision for the bulk culture (data not shown), supporting an overall lack of fusion in the PDPC culture system.

BM-Derived $Sca1^+$ Cells Cocultured with Pancreas *In Vitro* Do Not Acquire a PDPC Phenotype. To determine whether BM cells acquire a PDPC

20   phenotype because of coculture with pancreatic cells *ex vivo* or have acquired such a phenotype *in vivo*, Actb-GFP $Sca1^+$ BM cells (Figure 8B-a) were spiked at different concentrations in d7 islet-enriched fraction PDPC cultures (concurrent with PDPC appearance) derived from wild-type C57Bl/6 mice (n=6). During the ensuing week, GFP^+ cells began to proliferate and could be

25   found throughout colonies of PDPC-like cells (Figure 8A). On day 14, GFP^+CD45^+ cells were purified by FACS and analyzed by real-time qPCR (Figure 8C). Freshly isolated $Sca1^+$ BM cells expressed low levels of CKs and Afp, which remained relatively constant following 7 days of culture in a PDPC culture environment and remained significantly lower than levels detected in

30   PDPCs. Although levels of CK19 increased significantly (215-fold) by real-time RT-qPCR, this was only 15% of the level detected in $CD45^+$ PDPCs (Figure 8C), demonstrating that the PDPC phenotype is not acquired *ex vivo*.

Discussion

Presented herein is a protocol by which a population of oval cell-like progenitor cells residing in the normal pancreas can be expanded and purified *ex vivo*. Unlike the previous descriptions for isolation of hepatic or pancreatic oval cells (Jeffers et al. 1996; Petersen et al. 2003; Petersen et al. 1998; Wang et al. 2003), pancreas-derived progenitor cells (PDPCs) can be generated in the absence of previous chemical injury or damage and can be expanded *ex vivo*.

PDPCs proliferate as round or oval-shaped cells and express many markers similar to mouse hepatic oval cells including AFP, cMet, CKs 7, 8, 18, 19, CD34, CD45, Sca1, and Thy1(Knight et al 2005; Jeffers et al. 1996; Petersen et al. 2003; Petersen et al. 1998). Similar to oval cells, PDPCs produce numerous cytokines and growth factors including IL-6 and SCF, as determined by ELISA (data not shown) (Knight et al 2005; Fujio et al. 1996). Although CD45$^+$ PDPCs express the early endodermal marker AFP at levels much higher than a hepatic F2 oval cell-containing fraction, other epithelial markers are expressed at lower relative levels. The oval cell-containing fraction used in the studies described herein has been described as a size-purified (F2) primary cell fraction which appears during DDC-induced mouse liver oval cell activation (Wang et al. 2003). While PDPCs are uniformly CD45$^+$, some but not all cells within this population coexpress other stem cell markers including CD34, Sca1 and Thy1. Transcript levels for epithelial antigens in FACS selected CD45$^+$Sca1$^+$ cells were significantly higher than in the total CD45$^+$ fraction and approached those measured in the F2 liver fraction. This data suggests that subpopulations of CD45$^+$ PDPCs may have a more early endoderm progenitor phenotype (Sca1$^{-/low}$, CK^{low}) whereas others may represent a more committed epithelial progenitor phenotype (Sca1$^+$, CK^{high}).

As PDPCs resemble oval cells, it was tested whether they could be induced towards a more mature endodermal fate. Similar to mouse pancreatic oval cells, exposure of PDPCs to HGF/scatter factor induced a marked change in morphology (Jeffers et al. 1996). While purified PDPCs tend to form colonies or clusters of round cells, HGF treatment induced a flattened, epithelioid morphology. In addition, 4-6 weeks following application of the hepatic differentiation conditions, occasional lumen-like structures could be detected (data not shown). Real-time RT-qPCR demonstrated that PDPCs acquired a

more committed endodermal phenotype, expressing significantly higher levels of

epithelial markers, endoderm transcription factors, and pancreatic transcription

factors. Interestingly, as observed for cultured and primary mouse pancreatic

oval cells, PDPCs began to express mature hepatic markers including Albumin

5   even though they did not acquire a hepatocyte morphology (Ide et al. 1993).

Immunohistochemistry confirmed that progeny of PDPCs with epithelioid

morphology expressed Hnf3β.

.   While very few studies have evaluated pancreatic oval cells or their

origin, many studies have investigated the origin of hepatic oval cells (Wang et

10   al. 2003; Petersen et al. 1999; Menthena et al. 2004; Vig et al. 2006). Petersen et

al. (1999) suggested that, under certain pathological conditions, a bone marrow-

derived cell may act as a liver (oval cell) progenitor. While other groups have

reported similar results (Theise et al. 2000; Korbling et al. 2002), more recent

data suggest the majority of oval cells arise from within the liver itself, with less

15   than 1% of an exogenous BM origin (Wang et al. 2003; Menthena et al. 2004;

Vig et al. 2006). In addition, a significant portion of the BM-derived cells were

found to have fused with cells within the liver itself (Wang et al. 2003;

Menthena et al. 2004). The inventors and others have demonstrated that cells

can be cultured from rodent or human BM *ex vivo* and can subsequently be

20   induced to a hepatic (Schwartz et al. 2002) or pancreatic (D'Ippolito et al. 2004)

fate; however, it remains to be determined whether the long-term expansion

endows the BM cells with this differentiation potential. In addition, recent work

by Ruhnke et al (2005a; b) described the ability to differentiate short term *ex*

vivo expanded human monocytes to hepatic and pancreatic fates. Using a series

25   of lineage-tracing studies it was demonstrated herein that PDPCs do not

originate from a pancreatic endodermal cell or a beta cell, but that PDPCs are

derived from BM cells. Further characterization indicates that PDPCs are

derived from the KSL-enriched hematopoietic stem cell fraction in BM.

Although it cannot be excluded that only a small subpopulation of these cells

30   contribute to PDPCs, it was demonstrated herein that PDPCs are derived from

this stem cell enriched BM population. In addition, the data demonstrates that

PDPCs are not the product of fusion between BM-derived cells and endodermal

cells within the pancreas. Acquisition of the PDPC fate also does not appear to

be imposed by coculture between hematopoietic cells and pancreatic cells *ex*

vivo, as addition of Sca1$^+$ cells from BM to established PDPC cultures did not induce the endodermal phenotype seen in PDPCs. These results suggest that there exists a population of cells within the KSL fraction that has the potential to contribute to cells of non-hematopoietic lineages. As only 1:30 cells within the KSL population has been shown to possess long-term hematopoietic reconstitution potential (Osawa et al. 1996), the possibility exists that cells within this population have diverse potentials.

Current oval cell activation models in the rat (D-galactosamine (D-gal), retrosine/partial hepatectomy (Rs/PH), and 2-acetylaminofluorene/parital hepatectomy (2-AAF/PH)) or mouse (choline deficient, ethionine supplemented (CDE) diet or 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) diet) likely present a degree of damage repairable by endogenous epithelial stem/progenitor cells, without the need for a contribution from an exogenous source. As suggested by Knight et al. (2005), in the event that endogenous oval cells are exhausted, a BM population may serve as a last resort source. *Ex vivo* cultures likely present some of the signals required for the induction and proliferation of such exogenous oval cells.

Thus, PDPCs represent a novel, highly reproducible *ex vivo* expanded population of oval cell-like cells from an unmanipulated pancreas. PDPCs are believed to be derived from an exogenous bone marrow source and, like hepatic oval cells, coexpress hematopoietic and endodermal markers.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled

15   in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1.   A method of obtaining endodermal progenitor cells comprising:

(a)   culturing a cell population obtained from a tissue to induce cluster formation in culture medium;

(b)   culturing the clusters in culture medium; and

(c)   isolating endodermal progenitor cells,

wherein a function of a cell in the tissue is not reduced by application of an exogenous stressor prior to harvesting the tissue.

2.   The method of claim 1, wherein the tissue is endodermal tissue.

3.   The method of claim 1 or 2, wherein the tissue is derived from liver, stomach, intestine, pancreas, lung, colon, bladder or thyroid.

4.   The method of claim any one of claims 1-3, wherein the tissue is pancreas.

5.   The method of any one of claims 1-4, wherein the clusters are isolated prior to culturing in step (b).

6.   The method of any one of claims 1-5, wherein said clusters of step (b) are cultured with one more or attachment factors.

7.   The method of claim 6, wherein the one or more attachment factors comprise one or more of collagen type I, collagen type II, collagen type IV, fibronectin, chondroitin sulfate, vitronectin, thrombospondin, or matrigel.

8.   The method of any one of claims 1-7, wherein the culture medium of step (b) is changed every two to three days for at least about 7 days.

9.   The method of claim 8, wherein after about 7 days the culture medium of step (b) is not changed prior to isolating the endodermal progenitor cells.

10.   The method of any one of claims 1-9, wherein the culture medium of step (a) and/or (b) comprises serum.

5   11.   The method of claim 10, wherein the serum content is about 0.5% to about 5%.

12.   The method of any one of claims 1-11, wherein the culture media of step (a) and/or (b) comprises one or more of EGF, LIF, PDGF, or basic FGF.

10

13.   The method of any one of claims 1-12, wherein the cell population is subjected to continuous density gradient centrifugation prior to step (a).

14.   The method of any one of claims 1-13, wherein the tissue is disassociated
15   prior to step (a).

15.   The method of claim 14, wherein the tissue is disassociated enzymatically.

20   16.   The method of claim 15, wherein the enzyme comprises one or more of collagenase, trypsin, dispase I, hyaluronidase, thermolysin, neutral protease, liberase RI, DNase I, pancreatin, or pronase.

17.   The method of any one of claims 1-16, wherein the tissue is obtained
25   from a mammal.

18.   The method of claim 17, wherein the mammal is a human, swine, mouse or rat.

30   19.   An endodermal progenitor cell prepared by the method of any one of claims 1-18.

20.   The endodermal progenitor cell of claim 19, wherein the progenitor cell originates in bone marrow.

21.   A composition comprising a population of endodermal progenitor cells
       of claim 19 or 20 and a pharmaceutically acceptable carrier and/or
       culture medium.

5

22.   The method of any one of claims 1-18 further comprising differentiating
       the endodermal progenitor cells to yield an endodermal cell type.

23.   The method of claim 22, wherein the differentiated endodermal cell type
10   is selected from the group consisting of pancreatic, islet, intestinal,
       thyroid, lung, colon, bladder and liver cell types.

24.   The method of claim 22, wherein the endodermal progenitor cells are
       differentiated in the presence of one or more differentiation factors
15   comprising β-cellulin, GLP-1, HGF, FGF, EGF, KGF, nicotinamide,
       TGF-α, TGF-β, activin, cyclopamin, BMP4, an SHH antibody, FGF,
       EGF, Oncostatin M, dexamethasone, exendin4, GDF11, nicotinamide or
       a combination thereof.

20 25.   A method for providing an endodermal cell type to a subject in need
       thereof, comprising administering to said subject the endodermal
       progenitor cells produced by the method of any one of claims 1-18 or
       differentiated progeny derived therefrom in an amount effective to
       provide an endodermal cell type to the subject.

25

26.   The method of claim 25, wherein the endodermal progenitor cells or
       differentiated progeny derived therefrom are administered by systemic or
       local injection.

30 27.   An endodermal progenitor cell produced by the method of any one of
       claims 1-18 for use in medical therapy.

28.   The use of claim 27 wherein the medical therapy is treating pancreatic, intestinal, thyroid, lung, bladder or liver damage as a result of an injury or disease.

5   29.   The use of an endodermal progenitor cell produced by the method of any one of claims 1-18 to prepare a medicament for treating pancreatic, intestinal, thyroid, lung, bladder or liver damage as a result of an injury or disease.

10   30.   The use of claim 29, wherein the medicament includes a physiologically acceptable carrier and/or cell culture medium.
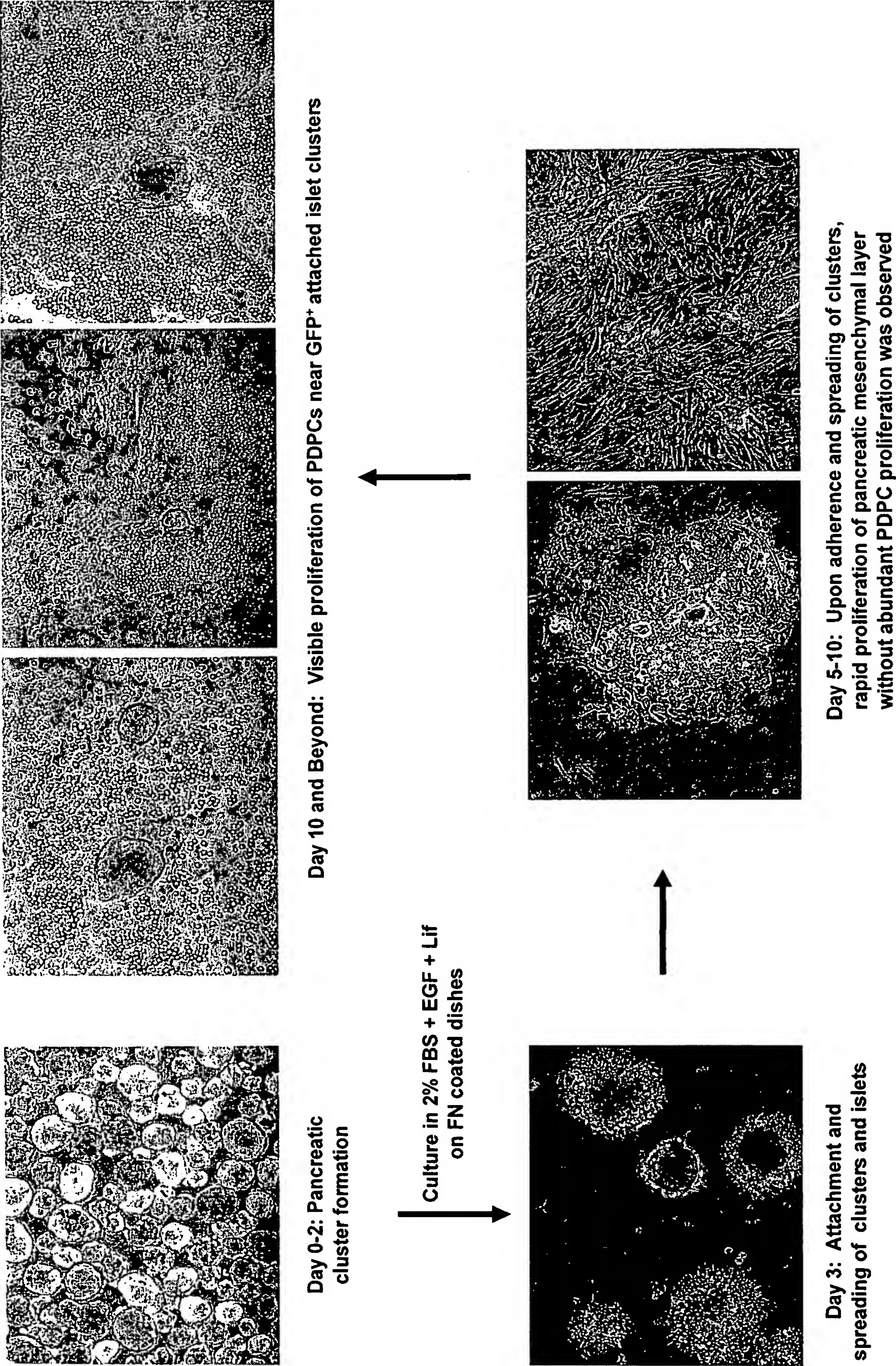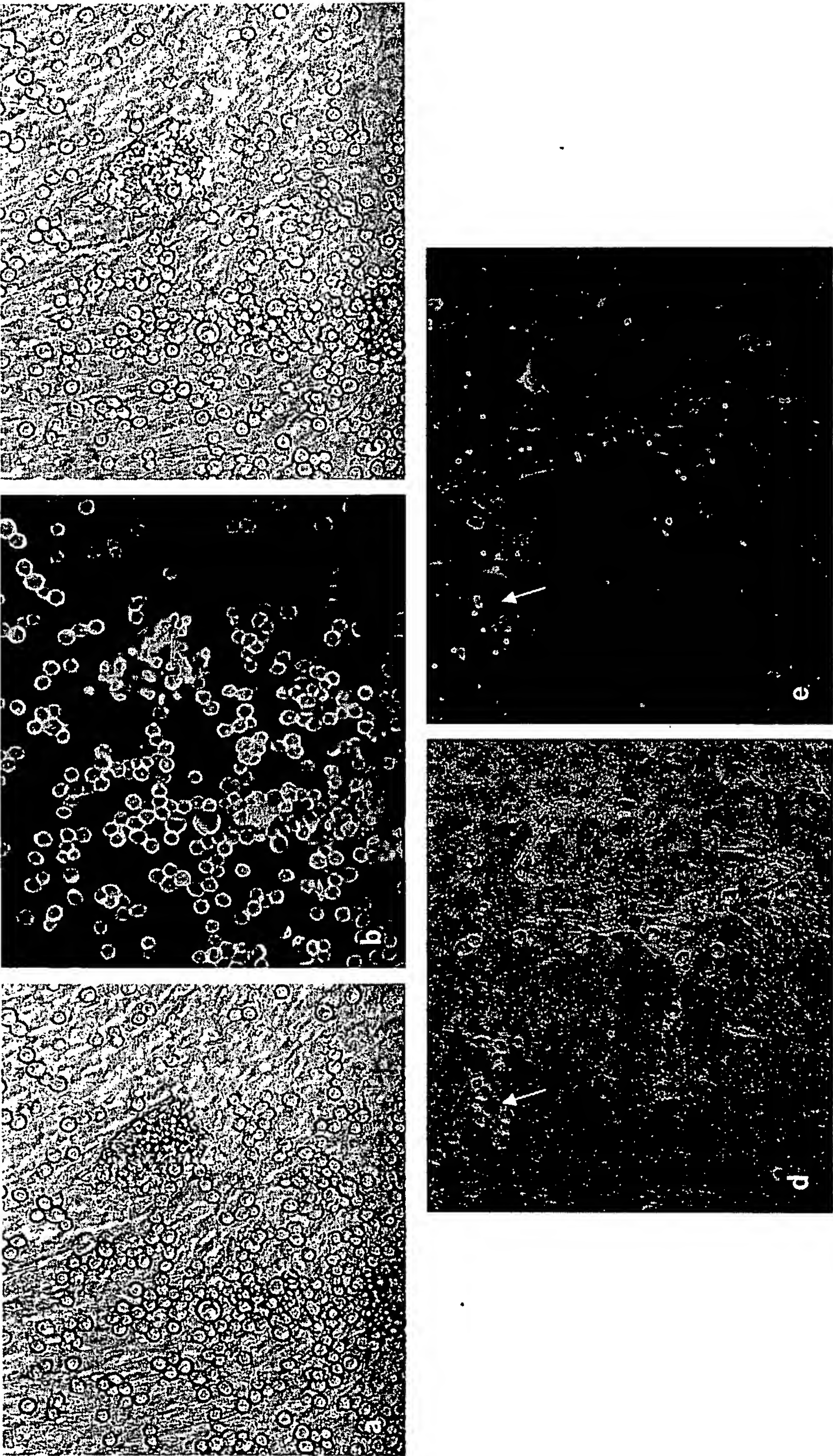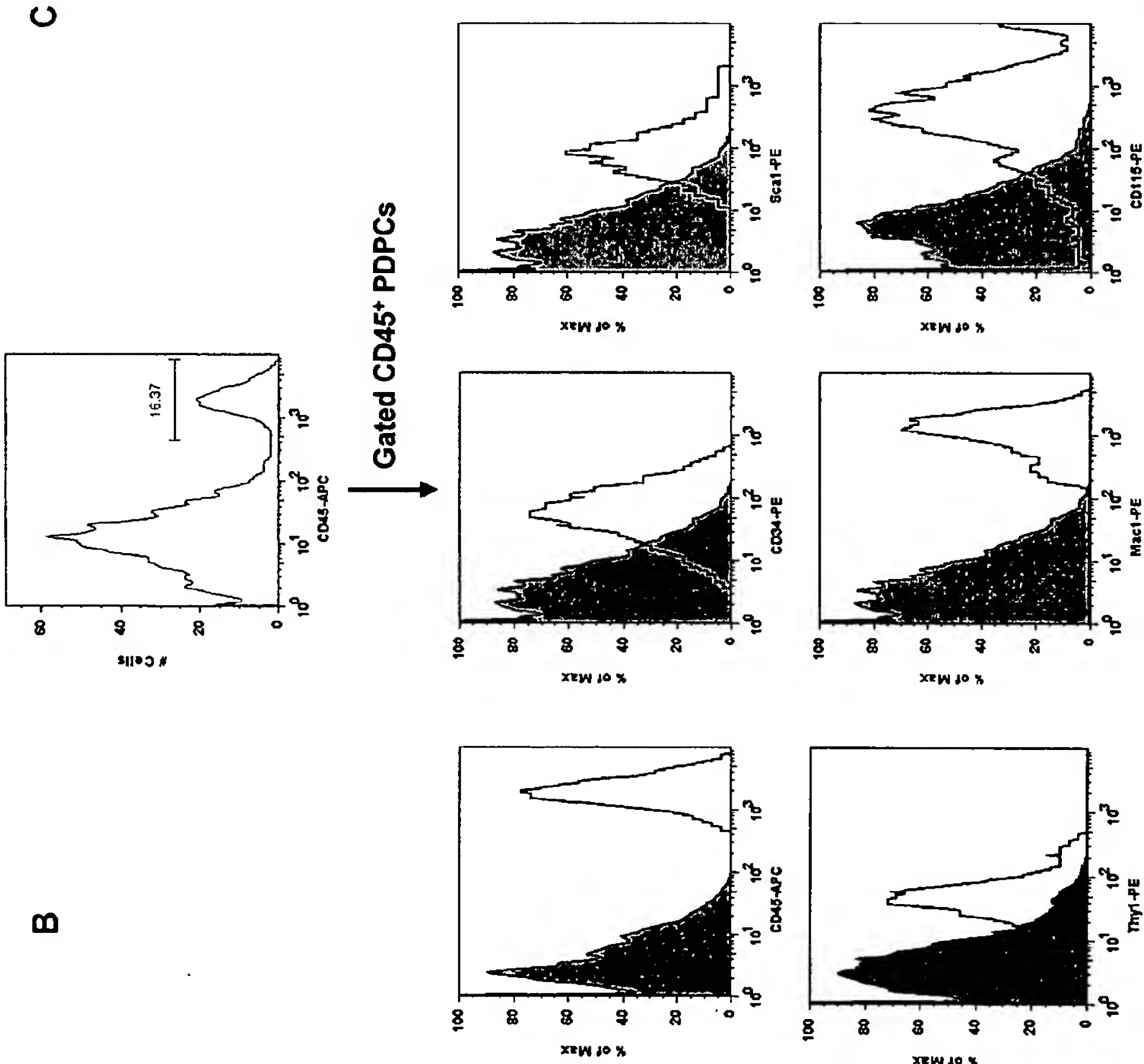
Day 10 and Beyond: Visible proliferation of PDPCs near GFP⁺ attached islet clusters

Day 0-2: Pancreatic cluster formation

Culture in 2% FBS + EGF + Lif on FN coated dishes

Day 3: Attachment and spreading of clusters and islets

Day 5-10: Upon adherence and spreading of clusters, rapid proliferation of pancreatic mesenchymal layer without abundant PDPC proliferation was observed

Figure 2.

Figure 3. Part (A).

C

		Target	ΔCt	CD45⁺ PDPCs Expression Level	% Relative Expression (CD45⁺ PDPCs E2⁰·ⁿI (Cell)
				Endoderm	
Early		Afp	9.25	+++	2965.08
		Sox7	11.95	+++	61.77
		Gata4	15.24	+	0.33
		Gata6	9.28	+++	150.52
Epithelial		CK7	11.27	+++	6.77
		CK19	12.28	+	1.68
		Cdh1(E)	10.93	+++	4.59
				Mesoderm	
Hematopoietic		Scl	12.13	+	123.54
		Lmo2	5.12	+++	597.94
		HoxB4	8.91	+++	145.90
		Runx1	6.33	+++	561.78
		Gata2	14.63	++	62.85
		Cxcr4	3.79	+++	188.56
		Kit	13.23	++	5.97
		PU1	4.76	+++	95.26
		Ikaros	6.67	+++	179.01
		Blnk	4.60	+++	3898.91
Endothelial		Flk1	9.64	+++	8.69
		Cdh5 (VE)	10.71	+++	1.62
		CD31	13.54	+	3.72
		VWF	7.35	+++	216.60
Other		Nestin	7.44	+++	332.88
		Vimentin	-0.17	+++	2200.87

Figure 3 Parts (B-D).

B

Gated CD45⁺ PDPCs

D

	Target	ΔCt CD45⁺/Sca1⁺ (1)	CD45⁺/Sca1⁻ (2)	Fold Change (2 vs. 1)	% Relative Expression (P=1 2⁰·ⁿI (Cell)	(1) (2)
FACS Purified PDPC Fractions						
	Actb	***	***	***	***	
	CK7	11.40	8.08	↑9.99	60.71	
	CK19	12.34	9.04	↑9.85	12.59	
	Cdh1	10.22	9.35	↑1.83	13.82	

A

B

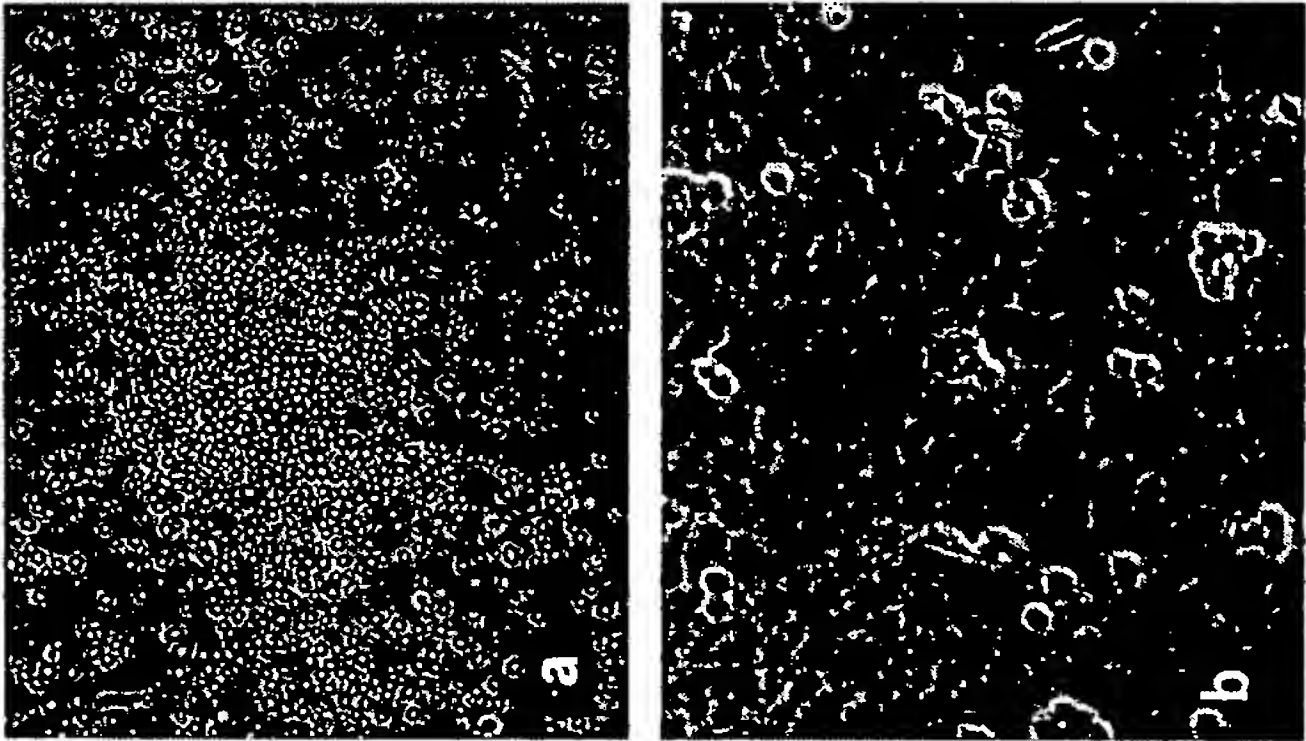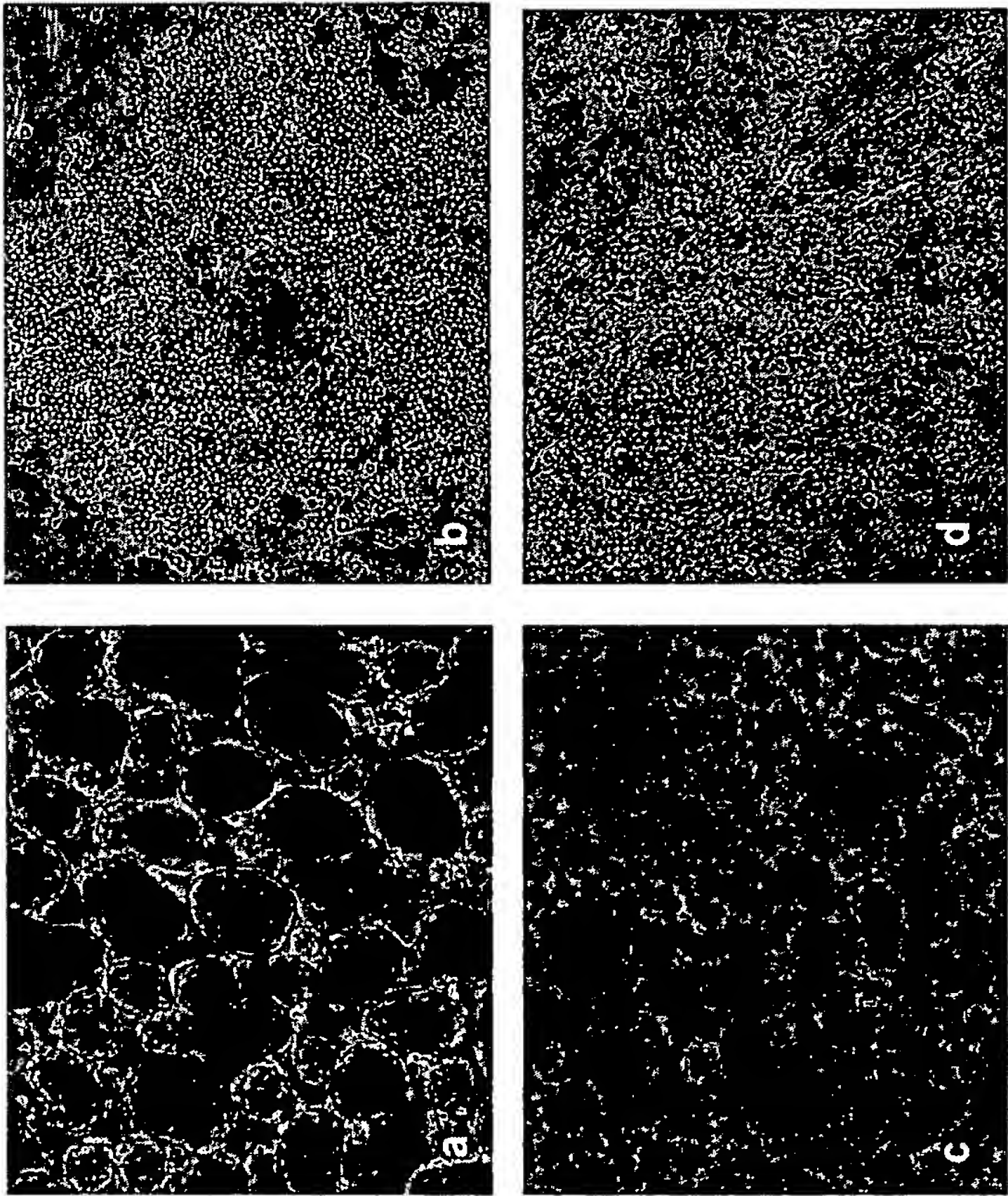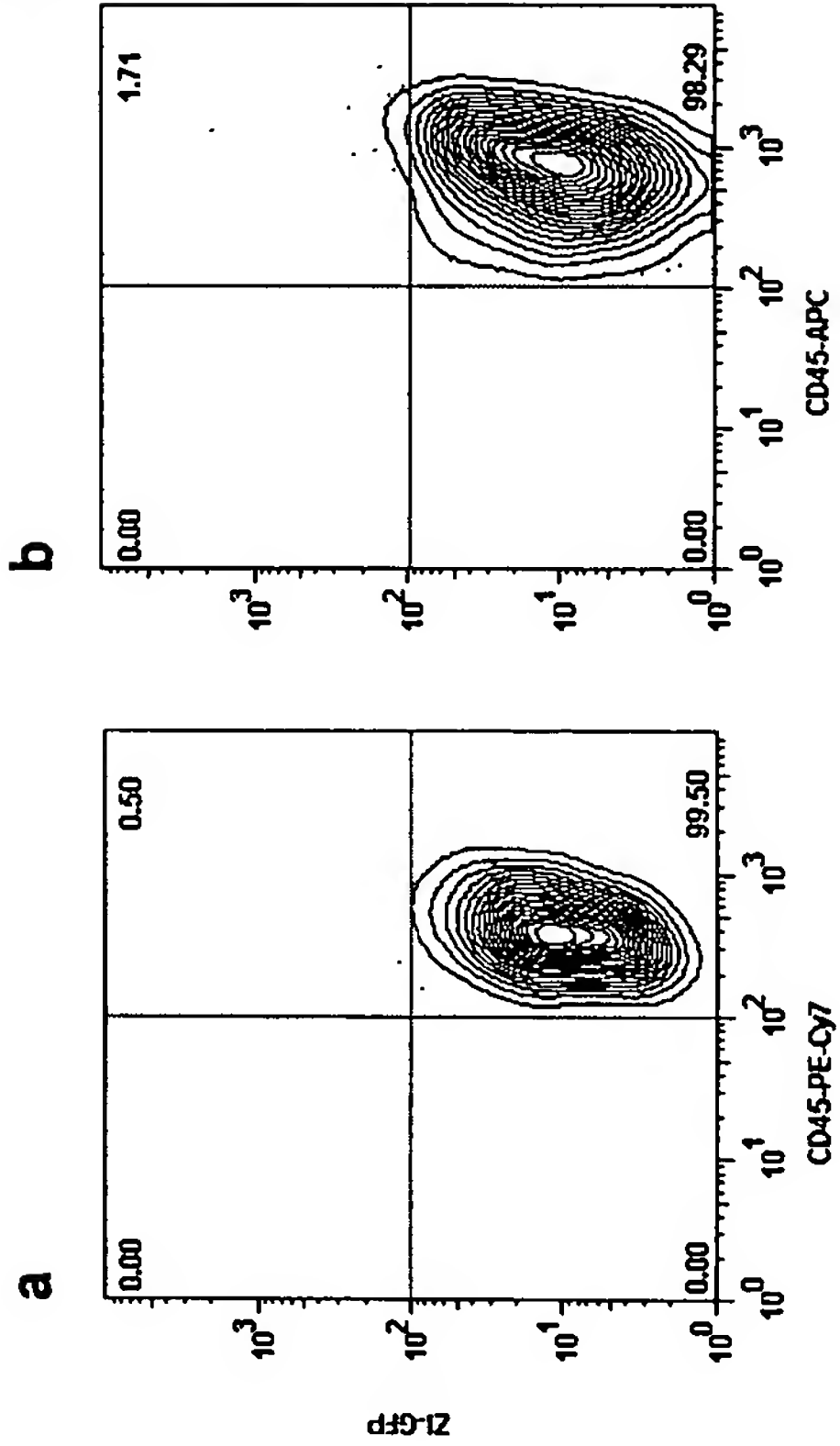| | Target | CD45+ PDPC (d0) | | PDPC Differentiation | | | | |
		Ct	Expression Level	Avg Ct	Std Dev	Expression Level	Regulation (Fold)	% Relative Expression (vs Mouse Total Liver)
Epithelial Markers	CK7	11.55	++	6.22	***	+++	↑40.22	3858.59
	CK8	10.78	++	6.48	1.75	+++	↑19.69	78.19
	CK18	8.94	+++	4.31	1.76	++++	↑24.76	64.62
	CK19	14.37	++	7.33	0.61	+++	↑131.59	555.97
	Cdh1	13.16	++	8.22	1.26	+++	↑30.70	55.48
	Car2	15.14	++	12.28	2.58	++	↑7.26	8.66
Endoderm Transcription Factors	Prox1	18.62	+	9.56	3.35	+++	↑533.74	3.54
	Gsc	NOT DETECTED		14.72	4.16	++	↑205.07	1487.69
	Sox17	14.33	++	11.20	0.84	+++	↑8.75	110.19
	Hnf1β	17.71	++	8.24	2.63	+++	↑709.18	152.10
	Hnf3α	NOT DETECTED		12.32	***	+	↑1082.39	7.13
	Hnf3β	NOT DETECTED		11.47	2.64	+++	↑1951.00	15.02
	Hnf4	16.60	++	8.42	***	+++	↑290.02	2.78
	Hnf6	NOT DETECTED		11.68	3.81	+++	↑1686.71	3.06
Pancreatic	Pdx1	15.10	++	9.61	***	+++	↑44.94	13.49*
	Nkx6.1	NOT DETECTED		9.35	***	+++	↑8480.89	20.67*
	INS2	14.08	++	17.06	***	++	↓7.89	0.00*
Hepatic	Afp	9.34	+++	14.04	0.47	++	↓26.00	1048.31
	Met	11.06	+++	7.53	***	+++	↑11.55	NOT TESTED
	Ttr	12.58	++	8.17	1.39	+++	↑21.26	0.20
	Alb	NOT DETECTED		13.94	0.94	++	↑352.14	0.00
	Cyp1a1	NOT DETECTED		9.57	***	+++	↑7281.40	665.76
	Cyp2b13	NOT DETECTED		9.29	2.11	+++	↑8841.04	0.78
	CD45	7.36	+++	9.87	***	+++	↓5.70	27.36

Figure 4 Parts (A) and (B).

Figure 4 Part (C).

Figure 5

Figure 6

Figure 7

Figure 8

Figure 1

Target	Sequence	Amplicon Size (bp)	SEQ ID NO:
Actb	F: TGTTACCAACTGGGACGACA		19
	R: GGGGTGTTGAAGGTCTCAAA	165	20
Afp	F: GCCCTACAGACCATGAAACAAG		21
	R: GTGAAACAGACTTCCTGGTCCT	149	22
CK7	F: CTCCCTGGACTTGGATGGTA		23
	R: ACGCTGGTTCTTCAAGGTGT	229	24
CK8	F:ATCGAGATCACCACCTACCG		25
	R:TGAAGCCAGGGCTAGTGAGT	151	26
CK18	F:CGAGGCACTCAAGGAAGAAC		27
	R:GCTGAGGTCCTGAGATTTGG	130	28
CK19	F:ACCCTCCCGAGATTACAACC		29
	R:CAAGGCGTGTTCTGTCTCAA	160	30
Cdh1	F:ACTGTGAAGGGACGGTCAAC		31
	R:GGAGCAGCAGGATCAGAATC	123	32
EpCAM	F:GCTGTCATTGTGGTGGTGTC		33
	R:CCCTCCTCAGTTCAGCACTC	165	34
Car2	F:TCAGGGAGCCCATTACTGTC		35
	R:GTTGTCCACCATCGCTTCTT	98	36
Prox1	F:GAAGGGCTATCACCCAATCA		37
	R:TGATGAGCTGCGAGGTAATG	133	38
Gsc	F:GAAGCCCTGGAGAACCTCTT		39
	R:ACCAGACCTCCACCTTCTCC	106	40
FoxD3	F:TACCCAATCCTGGACTCTGC		41
	R:GGACGGGTTGAGTTTGCTC	106	42
Sox7	F:TGTTGACAGATCCCCAAGAA		43
	R:CTGGGCACCAGTCAATTACA	130	44
Sox17	F:CTTTATGGTGTGGGCCAAAG		45
	R:CTTCTCTGCCAAGGTCAACG	121	46
Gata4	F:TCAAACCAGAAAACGGAAGC		47
	R:GCATCTCTTCACTGCTGCTG	146	48
Gata6	F:CAACACAGTCCCCGTTCTTT		49
	R:TGGTACAGGCGTCAAGAGTG	122	50
Hnf1β	F:CCGCAATCTCAGAACCTCAT		51
	R:AGGCTGCTAGCCACACTGTT	116	52
Hnf3α	F:ACAAGGATGCCTCTCCACAC		53
	R:ACCTCAGCATGACATGACCA	130	54
Hnf3β	F:CCCGGGACTTAACTGTAACG		55
	R:TCATGTTGCTCACGGAAGAG	152	56
Hnf4	F:TCTGCGAACTCCTTCTGGAT		57
	R:CCGAGGGACGATGTAGTCAT	137	58
Hnf6	F:CCCTGGAGCAAACTCAAGTC		59

	R:TTGGACGGACGCTTATTTTC	230	60
Pdx1	F:AACTTAACCTAGGCGTCGCA		61
	R:CATTCGCTTGGCATCAGAAGC	145	62
Nkx6.1	F:ACTTGGCAGGACCAGAGAGA		63
	R:AGAGTTCGGGTCCAGAGGTT	224	64
INS2	F:GACCCACAAGTGGCACAAC		65
	R:TCTACAATGCCACGCTTCTG	101	66
Nestin	F:AGGCTGAGAACTCTCGCTTG		67
	R:ATTAGGCAAGGGGGAAGAGA	167	68
Vimentin	F:ATGCTTCTCTGGCACGTCTT		69
	R:AGCCACGCTTTCATACTGCT	206	70
Ttr	F:CTTTGCCTCTGGGAAGACC		71
	R:CAGAGTCGTTGGCTGTGAAA	173	72
Alb	F:GACAAGGAAAGCTGCCTGAC		73
	R:TTCTGCAAAGTCAGCATTGG	174	74
Cyp1a1	F:ACCTCTTTGGAGCTGGGTTT		75
	R:GATAGGGCAGCTGAGGTCTG	165	76
Cyp2b13	F:CTGGCCACCATGAAAGAGTT		77
	R:GGGCTCCCTGGTATTTCTTC	106	78
CD45	F:TGAAAAGCAGCTGATGGATG		79
	R:AATACCCGTGGAATGCTCTG	123	80
PU.1	F:CGATCACTACTGGGATTTCTCC		81
	R:CAAGGTTTGATAAGGGAAGCAC	235	82
Lmo2	F:TACTACAAGCTGGGACGGAAAT		83
	R:ATGTCGGAGTTGATGAGAAGGT	212	84
Scl	F:GGAGATTTCTGATGGTCCTCAC		85
	R:AGTAACTTGGCCAGGAAATTGA	201	86
Runx1	F:TTCAGATGTGCATCGTGTCA		87
	R:CCAGCGGTTAGGCTTCATAC	104	88
Gata2	F:CAGGATGGGTGGAACATACTCT		89
	R:CATTTTGCTCTCCAAACAAACA	216	90
Cxcr4	F:TCCTGCCCACCATCTACTTC		91
	R:CCGTCATGCTCCTTAGCTTC	102	92
Ikaros	F:ATATTGTGGCCGGAGCTATAAA		93
	R:GCTCCTATCTTGCACAGGTCTT	159	94
Kit	F:TGGGAGTTTCCCAGAAACAG		95
	R:AAATGGGCACTTGGTTTGAG	149	96
Blnk	F:AGTCTGCTGAAGAGGCCTTG		97
	R:AACGCAACTAGGGTGTACGG	103	98
Flk1	F:TAAAGGCTCAGGCTGGTGTT		99
	R:TGGAGAGCAAACCAACCAAT	121	100
vWF	F:CCACTTGCCACAACAACATC		101
	R:TGGACTCACAGGAGCAAGTG	150	102
Cdh5	F:CTTCAAGCTGCCAGAAAACC		103
	R:ATTCGGAAGAATTGGCCTCT	129	104